Engulfment of Apoptotic Cell Corpses

in the Nematode *C. elegans*

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Zusammenfassung

Der programmierte Zelltod, auch Apoptose genannt, ist ein essentieller biologischer Prozess. Dieser spielt eine wichtige Rolle in der Embryonalentwicklung sowie in der Gewebehomöostase und reguliert die Immunantwort in vielen multizellulären Organismen. Um zu verhindern, dass der Inhalt einer apoptotischen Zelle in Kontakt mit dem umliegenden Gewebe kommt und dort eventuell Schäden verursacht, werden apoptotische Zellen von anderen Zellen erkannt, umschlossen und eliminiert. Dieser Prozess wird Engulfment oder Phagozytose genannt. Genetische Untersuchungen im Nematoden *C. elegans* führten zur Entdeckung von zwei unterschiedlichen, zum Teil überlappenden Signalwegen. Zusammen bewirken diese zwei Signalwege die effiziente Phagozytose von apoptotischen Zellen. Zu den Komponenten des einen Signalweges zählen der Rezeptor CED-1/CD91, der Adapter CED-6/Gulp, und der ABC Transporter CED-7/ABCA1. Der andere Signalweg besteht aus dem Adapter CED-2/CrkII, dem GEF-ähnlichen CED-5 /Dock180 Protein, der kleinen GTPase CED-10/Rac und dem CED-12/Elmo Protein. Man nimmt an, dass die Komponenten des zweiten Signalweges die Reorganisation des Aktin Zytoskelettes regulieren und eine Rolle in der Zellmigration spielen. Neben diesen relativ gut beschriebenen Proteinen ist jedoch nur wenig über weitere potentielle Aktivatoren und Effektoren der Signalkaskade bekannt.

In dieser Studie habe ich den Defekt von bereits bekannten Phagozytose Mutanten weiter untersucht, indem ich die Persistenz apoptotischer Zellen genau verfolgt habe. In verschiedenen Einzel- und Doppelmutanten untersuchte ich die Zeitspanne, die benötigt wird, um eine apoptotische Zelle zu eliminieren und verglich die unterschiedliche Kinetik der Zellelimination. Meine Daten bieten zusätzliche Evidenz für die Existenz weiterer Phagozytose Gene, die möglicherweise in einem dritten, bislang aber noch nicht identifizierten Phagozytose Signalweg eine Rolle spielen könnten.

Um neue Gene zu identifizieren, die spezifisch während der larvalen Entwicklung für die Phagozytose von apoptotischen Zellen benötigt werden, habe ich eine genetische Mutagenese in *ced-7* defekten Hermaphroditen durchgeführt. Durch diese Behandlung habe ich acht neue Mutationen isoliert, die einen Defekt in der Phagozytose verursachen. Unglücklicherweise ergab es sich, dass die von mir isolierten Mutanten neue Allele von bereits bekannten Phagozytose Genen waren. Da zu Beginn meiner Analyse jedoch nur zwei *ced-2* Allele bekannt waren, habe ich die zwei neuen Allele sequenziert. Beide Mutationen kodieren Unsinnmutationen und liegen am Ende des ersten Exons.

Im zweiten Teil meiner Arbeit habe ich die biochemischen Veränderungen auf der Zelloberfläche von apoptotischen Zellen untersucht. Solche Veränderungen sind wichtig für die Erkennung und die Elimination der apoptotischen Zelle durch die phagozytierende Zelle. In Vielzellern wird der Verlust der asymmetrischen Anordnung der Phospholipide in der Plasmamembran und das Vorhandensein von Phosphatidylserin (PS) auf der Zelloberfläche als charakteristischer Marker für apoptotische Zellen angesehen. Bis jetzt wusste man jedoch nicht, ob PS auch auf der Zelloberfläche von apoptotischen Zellen in *C. elegans* zu finden ist. Um diese Frage zu beantworten, habe ich transgene Würmer hergestellt, die ein induzierbares GFP::AnxV Reporter Konstrukt tragen (Annexin V ist ein Protein, das Phosphatidylserin bindet und in der Forschung häufig benutzt wird, um apoptotische Zellen nachzuweisen). Mit Hilfe dieses Reporters konnte ich zeigen, dass auch in *C. elegans* PS an die Zelloberfläche von apoptotischen Zellen transportiert wird.

Die Analyse von potentiellen Regulatoren dieses Prozesses führte zur Entdeckung, dass das Protein TAT-1, welches ein potentielles Homolog des Säugetierproteins ATPase II ist, eine wichtige Rolle in der Translokation von PS an die Zelloberfläche spielt. Das Ausschalten von *tat-1* durch RNAi bewirkte, dass in Embryos und in der Keimbahn von erwachsenen Hermaphroditen PS nicht mehr an die Zelloberfläche von apoptotischen Zellen transportiert wird und dass mehr apoptotische Zellen bestehen bleiben.

Diese Resultate zeigen, dass TAT-1 in der Translokation von PS an die Zelloberfläche von apoptotischen Zellen beteiligt ist und unterstützen die Hypothese, dass PS ein wichtiges Signal für die Phagozytose ist.

Zukünftige Untersuchungen werden helfen, die komplexen Mechanismen der Phagozytose aufzuklären. *C. elegans* ist ein viel erprobtes Modellsystem, welches zur Identifizierung und Charakterisierung von neuen Genen einen wertvollen Beitrag leisten kann.

Summary

Apoptosis or programmed cell death is an essential process that plays a fundamental role in development, tissue homeostasis, and defence of many multicellular organisms. In order to prevent leakage of potentially harmful intracellular contents into the surrounding tissues, apoptotic cells are recognised and rapidly eliminated by other cells, a process termed engulfment. Genetic studies in the nematode *C. elegans* have identified two distinct, partially redundant pathways that act synergistically to promote the engulfment of apoptotic cells. Components of one pathway include the receptor CED-1/CD91, the adaptor protein CED-6/Gulp, and the ABC transporter CED-7/ABCA1. The other pathway consists of the adaptor protein CED-2/CrkII, the GEF-like protein CED-5/Dock180, the small GTPase CED–10/Rac1, and the CED-12/Elmo protein. Members of the second pathway are thought to regulate the reorganisation of the actin cytoskeleton and play a role in cell migration processes. However, besides those rather well-described proteins, little is known about upstream activators and downstream effectors of the engulfment signalling machinery.

In this study, I further analysed the engulfment defect of the known engulfment mutants by performing a thorough time course analysis of cell corpse persistence. I scored unengulfed cell corpses in all single and many different double mutants and compared the kinetics of cell corpse removal between the distinct mutants. My data presents further evidence for the existence of additional engulfment genes that might act in a third, as yet unidentified, engulfment pathway.

In order to identify novel engulfment genes, which specifically promote engulfment during larval development, I performed a genetic screen in a sensitised *ced-7* mutant background. Using this approach, I isolated eight new mutations that result in defective phagocytosis. Unfortunately, they all turned out to be novel alleles of already known engulfment genes. Since there were only three alleles of *ced-2* known prior to my screen, I sequenced the two new

alleles of *ced-2*. Both alleles are missense mutations located at the end of the first exon of CED-2.

In the second part of my work, I assessed the biochemical changes occurring at the cell surface of apoptotic cells that might play a role in the recognition of the apoptotic cell by the phagocyte. Loss of plasma membrane phospholipid asymmetry and subsequent exposure of phosphatidylserine (PS) has been considered a hallmark of apoptotic cells in metazoans. However, it was not known so far whether apoptotic cells in *C. elegans* expose PS as well. To answer this question, I generated transgenic animals expressing an inducible GFP::AnxV reporter construct in *C. elegans*. Using this in vivo approach, I found that dying cells in *C. elegans* likely expose PS, as shown by the selective binding of the GFP::AnxV protein to the surface of apoptotic cell corpses in embryos and the germ line of adult hermaphrodites.

Analysis of potential regulators of PS exposure on apoptotic cells in *C. elegans* revealed an important function for TAT-1, the worm homologue of mammalian ATPase II, in this process. Ablating the function of *tat-1* by RNA interference (RNAi) impaired PS exposure on apoptotic cells and increased the number of unengulfed apoptotic cell corpses in embryos and the adult hermaphrodite germ line. These results implicate a role for TAT-1 in the translocation of PS across the plasma membrane in cells undergoing apoptosis and support the hypothesis that PS acts as an ‘eat-me’ signal for engulfing cells in *C. elegans*.

Future research will help to shed more light into the complex mechanisms of engulfment. For this purpose, *C. elegans* has proved to be a powerful model organism, making the identification and characterisation of novel genes a feasible venture.

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Chapter 1

Introduction

1.1. *C. elegans* as a Model Organism

Since its establishment as a model organism in 1965 by Sydney Brenner, the nematode *Caenorabditis elegans* has been proved to be a powerful genetic model system for studying several aspects of developmental biology and neurobiology [1]. The characteristics that make *C. elegans* an attractive experimental model include its short life cycle (3-4 days at 20°C), its small size (adult hermaphrodites are only 1mm in length) and its facile propagation and maintenance in the laboratory. The main sex of *C. elegans* is the self-fertilising hermaphrodite, which is capable of producing approximately 300 progeny. Each embryo will develop, hatch, and proceed through four larval stages before becoming an adult [2]. Males arise spontaneously at low frequency (0.1%) and can cross-fertilise hermaphrodites. *C. elegans* is transparent and has a simple body plan, allowing observation of individual cells in living worms using Nomarski optics. The complete cell lineage has been determined by observation of cell divisions and cell migrations in developing wild-type animals [3,4]; this lineage turned out to be essentially invariant. The adult hermaphrodite consists of only 959 somatic cells, yet containing many different cell types such as neurons, muscles, intestine, and epidermis. Importantly, major signal transduction pathways are evolutionarily conserved between *C. elegans* and mammals, making *C. elegans* an attractive model organism to study human disease-related genes.

The nematode *C. elegans* provides a powerful genetic tool that allows the isolation of novel genes by forward mutagenesis. Isolated mutants can be analysed and characterised using standard, well-established methodologies. Reverse genetic analysis of an individual gene of interest can be performed by *RNA* mediated *i*nterference (RNAi) or mutagen-induced production of deletion alleles [5,6]. In addition, the complete sequenced genome [7] provides an enormous amount of data to further study *C. elegans* biology taking advantage of newly developed technologies such as microarrays and comparative genomics.

1.2. Programmed Cell Death

Apoptosis or programmed cell death plays a fundamental role in metazoan development, metamorphosis and tissue homeostasis of the adult animal [8]. Disturbance of the apoptotic program can contribute to various disease states, including cancer, neurodegenerative disorders, and acquired immunodeficiency syndrome [9].

The nematode *C. elegans* is an attractive model organism for studying apoptosis on account of its strict cell lineage, its powerful genetics and its complete sequenced genome. Apoptosis is a common cell fate in *C. elegans*: Of the 1090 cells generated during development of the *C. elegans* hermaphrodite, 131 die by undergoing apoptosis [4,10-12]. In addition, in the adult hermaphrodite germ line almost half of all germ cells are eliminated by programmed cell death [13]. The pattern of developmental programmed cell death is essentially invariant and the identity and timing of each cell death is known [10]. Genetic studies have identified at least 15 genes that affect the process of programmed cell death in *C. elegans*. Based on the order of their activity, these 15 genes can be divided into four functional groups: (I) decision to die, (II) death execution, (III) engulfment and (IV) DNA degradation (Figure 1). (I) *ces-1* (*ce*ll death *s*pecification) and *ces-2* have been found to regulate the death of specific cells, the NSM sister cells, and genetic studies have shown that both genes act upstream in the apoptotic pathway [14-16]. *eor-1* and *eor-2* (*e*gl-1 suppressor, Di*O* uptake defective, *r*af enhancer) function specifically to promote cell death of the HSN neurons in males [17]. (II) Two genes, *ced-3* (*ce*ll *d*eath abnormal) and *ced-4*, are absolutely required for all programmed cell deaths: if the function of either gene is lost, no programmed cell death occurs and all cells survive [18]. A third gene, termed *ced-9*, has been shown to prevent programmed cell death and was initially identified by a gain-of-function mutation *n1950*, which dominantly blocked all somatic cell death [19]. (III) Apoptotic cells are recognised and rapidly engulfed by neighbouring cells. At least seven genes are required for the efficient removal of dying cells, namely *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10* and *ced-12* [20-22]. (IV) The

final phase of the apoptotic program comprises the fragmentation and degradation of chromosomal DNA [23]. NUC-1 (*nuc*lease abnormal), the homologue of mammalian DNase II and CPS-6 (*C*ED-3 *p*rotease *s*uppressor 6), the orthologue of mammalian EndoG have been shown to mediate DNA degradation in apoptotic cells [24-26]. Recently, WAH-1 (*w*orm *A*IF *h*omologue 1), a worm homologue of AIF (*A*poptosis *I*nducing *F*actor), was found to associate with and cooperate with CPS-6 to promote DNA degradation in *C. elegans* [26].

1.2.1. Molecular Analysis of the Apoptotic Pathway in *C. elegans*

The Core Apoptotic Machinery

Essentially, all programmed cell deaths are mediated by the core apoptotic machinery, which includes the caspase CED-3 [27], the Apaf-1 homologue CED-4 [28], and the Bcl-2 family member CED-9[19] (Figure 2). CED–9 prevents cell death by antagonising the death-promoting activities of CED-3 and CED-4 [19,29]. In living cells, CED-3 exists as an inactive proenzyme, likely bound in a ternary complex with CED-4 and CED-9, localised to the surface of mitochondria [30-32]. Following a pro-apoptotic stimulus, the BH3 domain protein EGL-1 binds to the hydrophobic pocket of CED-9, inducing a conformational change in CED-9, which allows CED-4 to dissociate from the complex [33-35]. Dissociation of CED-4 promotes the processing of CED-3, which cleaves as yet unknown caspase substrates in the cell, thereby triggering apoptosis. In the absence of functional CED-3 or CED-4, no programmed cell death occurs [18].

ced-9

ced-9 is the *C. elegans* homologue of the mammalian proto-oncogene *bcl-2* and functions to prevent apoptosis [19,36]. The mammalian Bcl-2 family of proteins includes pro-apoptotic as well as anti-apoptotic proteins. Members of the Bcl-2 family are characterised by 1-4 conserved Bcl-2 homology (BH) domains, roughly corresponding to α helices, which dictate structure and function. CED-9 has been shown to biochemically interact with CED-4 [37] and

to regulate the subcellular localisation of CED-4, causing both molecules to localise to the mitochondria [32]. In living cells, CED-9 is tightly bound to CED-4, thereby preventing its oligomerisation. In cells doomed to die, the pro-apoptotic protein EGL-1 binds to CED-9 and causes the disruption of the CED-9/CED-4 complex.

ced-4

Mutations in *ced-4* abrogate almost all programmed cell deaths that normally occur during *C. elegans* development [18]. *ced-4* encodes the homologue of mammalian Apaf-1, an adaptor molecule containing 12 WD-40 repeats [27,28,38]. In mammalian cells, Apaf-1 has been shown to bind cytochrome *c* via its WD-40 domain. Upon binding to cytochrome *c*, Apaf-1 becomes competent to activate caspase-9 in the presence of ATP/dATP. This interaction is mediated by caspase recruitment domains (CARD) present in both Apaf-1 and caspase-9 [39]. *C. elegans* CED-4 does not have any WD-40 repeats and hence cannot bind cytochrome *c*. In healthy cells, CED-4 is bound by CED-9 and kept in an inactive conformation. However, in cells fated to die, binding of the pro-apoptotic protein EGL-1 to CED-9 displaces CED-4, which then translocates to the perinuclear region [32]. Released CED-4 undergoes oligomerisation, thereby promoting autocatalytic activation of CED–3 by an induced proximity mechanism [40,41].

ced-3

Loss-of-function mutations in *ced-3* completely abrogate programmed cell death in *C. elegans* [18]. *ced-3* encodes a protein related to the mammalian interleukin 1β converting enzyme (ICE) [27], the founding member of the caspase family of proteases (caspase-1). Caspases are produced as inert zymogens, which have to be activated by dimerisation (initiator caspases) or proteolytic processing (effector caspases). Many caspases contain a conserved CARD (*ca*spase *r*ecruitment *d*omain) domain, which mediates the association of adaptor proteins and procaspases through heterodimerisation, recruiting procaspases to upstream signalling complexes and allowing their autoactivation. In *C. elegans*, activated CED-3 cleaves as yet unknown downstream targets that induce DNA fragmentation and degradation. Until

today, only a few CED-3 substrates have been described: notably CED-3 itself, the caspase homologue CSP-2B, CED-9, the baculovirus protein p35, and FEM-1, a protein involved in regulating sex determination [40,42-44]. FEM-1 (*fem*inisation of XX and XO males 1) negatively regulates TRA-1A (*tra*nsformer 1A), which represses the transcription of the pro-apoptotic gene *egl-1* [45] in the HSN (*h*ermaphrodite-*s*pecific *n*eurons) in hermaphrodites. In addition, FEM-1 has been shown to bind CED-4 [44].

egl-1

Dominant gain-of-function mutations in *egl-1* were originally isolated in genetic screens for *C. elegans* hermaphrodites defective in egg laying. Closer examination of the *egl-1*(gf) phenotype revealed that the HSN neurons inappropriately undergo apoptosis in these animals [46]. Normally, programmed cell death of the HSN neurons is prevented in hermaphrodites by the function of *tra-1*, the terminal global regulator of somatic sex determination [47]. The *egl–1*(gf) mutations disrupt the TRA-1A binding site 5.6kb downstream of the *egl-1* transcription unit, resulting in inappropriate activation of the *egl-1* gene in the HSN neurons in hermaphrodites [45]. *egl-1* encodes a protein that contains a region similar to the BH3 domain of Bcl-2-like and BH3-containing proteins and interacts biochemically with CED-9. A loss-of-function mutation in *egl-1* prevents all somatic cell deaths, indicating that EGL-1 is a positive regulator of the apoptotic pathway [33]. Binding of EGL-1 to CED-9 promotes the dissociation of CED-9 from CED-4 and allows CED-4-dependent processing of CED-3 [34,35].

1.2.2. Genes Involved in Cell-Specific Cell Death

ces-1, ces-2

In *C. elegans*, *ces-1* and *ces-2* control the decision of two specific cells to die by apoptosis [16]. Gain-of-function mutations in *ces-1* and loss-of-function mutations in *ces-2* prevent the sisters of the NSM neurons from undergoing programmed cell death. Mutations in *ces-1* also prevent the sisters of the I2 neurons from dying. Importantly, other cell deaths are not affected by either

mutations in *ces-1* or *ces-2*. Genetic studies indicate that *ces-1* and *ces-2* control the death of the sisters of the NSM neurons by regulating the genes that act in all cell death (i.e. *ced-3*, *ced-4*). *ces-2* encodes a basic region leucine-zipper (bZIP) transcription factor and is believed to function as a negative regulator, possibly as a transcriptional repressor of a gene required for survival such as *ces-1* [15]. Indeed, CES-2 has been shown to bind to an upstream element of *ces-1* and mutations in this regulatory element render *ces-1* insensitive to *ces-2* activity, supporting the hypothesis that CES-2 directly suppresses the transcription of *ces-1*. *ces-1* encodes a snail family zinc finger protein and might negatively regulate a transcriptional cascade promoting the death of specific pharyngeal neurons [14]. Recently, CES-1 was found to bind to the Snail-binding sites/E-boxes of the *egl-1* locus, where it competes for binding with HLH-2/HLH-3, a direct, cell-type specific activator of *egl-1* transcription [48].

eor-1, eor-2

eor-1 and *eor-2* have recently been described to play a role in the specification of cell death [17]. Multiple genetic screens isolated *eor-1* and *eor-2* as suppressors of *egl-1*, enhancers of *lin-45/raf*, as well as having defects in phasmid neuron dye uptake [49,50]. Loss-of-function mutations in *eor-1* or *eor-2* prevent HSN death in *egl-1*(gf) hermaphrodites and wild-type males, demonstrating that *eor-1* and *eor-2* are required to specify HSN death. In addition to impaired HSN death, *eor-1* and *eor-2* mutants display a common set of pleiotrophic defects throughout the nervous system, consistent with defects in neuronal cell differentiation. Although the identities of both genes are known, with *eor-1* encoding a putative transcription factor, related to the human oncogene PLZF and *eor-2* encoding a novel but conserved protein, the particular mode of action of *eor-1* and *eor-2* remains currently unknown.

1.2.3. Genes Involved in DNA Degradation

nuc-1

A hallmark of apoptosis is the cleavage of chromosomal DNA into oligonucleosome-sized fragments, a process known as DNA fragmentation [23]. *C. elegans nuc-1* is required for DNA degradation but is not involved in either apoptosis or engulfment of apoptotic cell corpses [51]. In *nuc-1* mutants, the pycnotic DNA of dead cells is not degraded and persists as a compact mass [20,51]. Consistent with its role in DNA degradation *nuc*-1 mutant worms are also defective in digestion of bacterial DNA in the gut lumen [51]. Importantly, *nuc-1* encodes an acidic nuclease with significant homology to mammalian DNase II [25,52].

cps-6

In addition to NUC-1, another nuclease, CPS-6 has been reported to play an important role in DNA fragmentation [24]. *cps-6* encodes a homologue of human endonuclease G (endoG) and its protein product similarly localises to mitochondria. Interestingly, *cps-6* has been identified as a gene that, when mutated, suppresses the apoptotic activity of activated CED-3 [24]. In other words, CPS-6 not only promotes DNA degradation, but also appears to contribute to cell death. Considering previous studies, suggesting that DNA degradation is a late, non-essential step in apoptosis, these findings are rather surprising. Perhaps CPS-6 acts at an early step in the apoptotic program, when the cell's fate still can be converted from death back to life.

wah-1

A second hint for a role of mitochondria in programmed cell death in *C. elegans* came from studies of *wah-1*, the worm homologue of AIF [26]. *wah-1* RNAi causes a delay in cell death progression and a defect in apoptotic DNA degradation in *C. elegans* embryos. WAH-1 localises to mitochondria and is released into the cytosol and nucleus by *egl-1* expression in a caspase CED-3 dependent manner. Notably, GST-pull-down experiments demonstrated that WAH-1 and CPS-6 interact and in vitro cleavage studies suggest that WAH-1

and CPS-6 cooperate to promote apoptosis and DNA degradation, providing further evidence that *wah-1* and *cps-6* act in the same genetic pathway.

crn-1

crn-1 (*c*ell death *r*elated *n*uclease 1), a *C. elegans* homologue of FEN-1 (*f*lap *e*ndo*n*uclease 1), which is normally involved in DNA replication and repair [53], displays similar functions as *cps-6*. *crn-1* RNAi-treated worms throw embryos that are defective in DNA degradation and seem to have a delay in timely progression of developmental cell death [54]. Treating either partial or strong *ced-3* and *ced-4* loss-of-function mutants with *crn-1* RNAi, enhances the cell death defect, suggesting that CRN-1 promotes apoptosis. Interestingly, *crn-1* RNAi does not enhance the DNA degradation or cell killing defect caused by CPS-6, indicating that *crn-1* and *cps-6* act through the same pathway. In vitro cleaving assays suggest that CRN-1 has similar nuclease activities as FEN-1, enhancing the nuclease activity of CPS-6. In addition, CRN-1 interacts with CPS-6, supporting the idea that CRN-1 acts as a co-factor of CPS-6 in DNA degradation and cell killing.

A candidate-based RNAi screen, performed by Parrish and Xue (2003), identified seven additional genes that appear to be required for DNA degradation and apoptosis [55]. The genes examined encode deoxyribonucleases and ribonucleases as well as cyclophilins and topoisomerases and homologues of all of them are found in the mammalian genome. Although immunoprecipitation studies grouped the CRN nucleases into multiple DNA degradation complexes, it is not known yet, whether this degradeosome has any biological function in vivo.

1.2.4. Apoptotic Cell Death, Independent of *ced-3* or *ced-4*

icd-1

Loss of *icd-1* (*i*nhibitor of *c*ell *d*eath) results in increased apoptosis during *C. elegans* development [56]. Although this cell death requires CED-4, it occurs independently of the caspase CED-3. ICD-1 is homologous to

mammalian βNAC (*β*-subunit of the *n*ascent polypeptide-*a*ssociated *c*omplex) and contains a putative caspase-cleavage and a caspase-recruitment domain. However, the target caspase of ICD-1 has not been identified yet, as CED-3, the only caspase involved in apoptosis in *C. elegans*, is not required for *icd-1* RNAi induced cell death. ICD-1 localises mainly to mitochondria, emphasizing the role of mitochondria in coordinating apoptosis. But how *icd-1* protects cells from undergoing programmed cell death remains to be determined.

pvl-5

Recently, the product of *pvl-5* (*p*rotruding *v*u*l*va) has been found to protect hypodermal cells from undergoing programmed cell death in *C. elegans* [57]. *pvl-5* mutant animals have fewer vulva precursor cells (P.n.p.'s), causing vulva induction defects that result in a protruding vulva phenotype. Surprisingly, these extra cell deaths are only dependent on *ced-3* and *ced-9*, but not on *egl-1* or *ced-4* function. As the *pvl-5* gene has not been cloned yet, it remains unknown what kind of protein *pvl-5* encodes. Certainly, future studies will provide further insights into the phenomena of programmed cell deaths that deviate from the established core apoptotic pathway.

1.2.5. Programmed Cell Death in the Adult Hermaphrodite Germ Line

The *C. elegans* germ line consists of two symmetrical U-shaped tubular arms that converge at a common uterus (Schematic drawing of one gonad arm is shown in Figure 3). At the distal end of each gonad arm resides the distal tip cell (DTC) that promotes mitotic germ cell proliferation. Germ cells positioned beyond the influence of the proliferating signal provided by the DTC enter meiosis and progress into the pachytene stage of meiosis I. Upon activation of the Ras/MAPK pathway, near the bend of the gonadal tube, cells progress to diakinesis and differentiate into mature oocytes. At this stage of germ cell maturation more than half of all germ cells die by apoptosis [13]. It has been shown that physiological germ cell death is dependent on the core apoptotic machinery: *ced-3*, *ced-4* and *ced-9*. However, a rare gain-of-function mutation in *ced-9*, which completely prevents all somatic cell deaths,

has little effect on germ cell deaths. Interestingly, *egl-1*(lf) does not affect germ cell apoptosis, indicating that activation of the apoptotic machinery is regulated differently in the soma and the hermaphrodite germ line.

So far, only a few genes have been described to affect physiological germ cell death in *C. elegans*. *daz-1*(*d*eleted in *az*oospermia 1) encodes a ribonucleoprotein-like RNA binding protein required for oogenesis. In *daz-1*(lf) hermaphrodites oogenesis is blocked at the pachytene stage of meiosis I and the arrested germ cells undergo apoptosis through as yet unknown mechanisms [58]. *cgh-1* (*c*onserved *g*erm line *h*elicase 1) encodes a predicted RNA helicase that appears to function especially in the germ line [59]. Knocking down the function of *cgh-1* by RNAi results in increased germ cell death and the production of non-functional sperm. However, it is not understood yet how *cgh-1* protects germ cells from undergoing apoptosis.

Recently, another set of *g*erm *l*ine *a*poptosis genes, named the *gla* genes, has been identified in the Hengartner laboratory (Milstein and Hengartner, *unpublished*). In *gla*(lf) mutant worms physiological germ cell deaths are enhanced and these cell deaths have been shown to depend on the core apoptotic machinery. Intriguingly, two of the *gla* genes, *gla-1* and *gla–3*, encode putative RNA binding proteins and one can imagine that they might regulate the translation of pro-apoptotic mRNA targets. However, the process of physiological germ cell death is far from being understood and further studies are definitively required to elucidate the mechanisms of programmed cell death in the *C. elegans* germ line.

1.2.6. Apoptosis vs. Necrosis

Apoptosis and necrosis are two morphologically distinct types of cell death and apoptotic cells appear substantially different from necrotic cells [23]. Apoptotic cell death is characterised by condensation of the cytoplasm and nucleus, resulting in a high refractile appearance of the dying cell. In this process the cellular organelles maintain their integrity and the DNA degrades

into nucleosome-sized fragments. On the other hand, necrotic cells lose their membrane integrity and the cellular organelles are disrupted. As a consequence the cells swell extensively and undergo cell lysis.

In *C. elegans*, necrotic-like cell death can be induced by gain-of-function mutations in a number of specific ion channel genes, such as the degenerin genes *deg-1* and *mec-4* [60,61], the acetylcholine receptor channel subunit gene *deg-3* [62] and the G(s) protein alpha-subunit gene *gsa-1* [63,64]. The death inducing substitutions in these proteins are thought to hyperactivate the channels, resulting in increased or altered ion flow and/or imbalanced osmotic balance, culminating in cell death. Importantly, necrotic cell death only affects neuronal cells and is not dependent on *ced-3* or *ced-4*, but clearance of necrotic corpses requires the same engulfment machinery than apoptotic corpses [65]. These results imply that a common set of engulfment genes is required to remove apoptotic and necrotic cells, possibly activated through the same 'eat-me' signal on both corpse types. Regardless of this similarity, there must be differences between the elimination of apoptotic and necrotic cell corpses. One significant difference is the time required to complete corpse removal. Apoptotic corpses are swiftly engulfed in less than one hour, [4] whereas necrotic corpses often linger for several hours [60,66]. It is not clear yet why engulfment of necrotic cell corpses takes so much longer, but since necrotic cells are bigger in size than apoptotic cells, one can speculate that eating a bigger meal simply requires more time. Alternatively, distinct kinetics might reflect differences in the recognition of apoptotic and necrotic cells.

1.2.7. Future Directions

In the last ten years of biological research, enormous effort has been undertaken to elucidate the mechanisms of programmed cell death. Multiple studies in *C. elegans* have contributed to our current knowledge of programmed cell death in worms and mammals, but the apoptotic puzzle is far from being complete. Various key players of the apoptotic pathway remain elusive: what is the upstream signal(s) lightening the apoptotic cascade?

What molecules transduce the apoptotic signal(s)? Inevitably, further studies are required to elucidate the mysteries of programmed cell death. To fulfil this task, *C. elegans* still represents a favourable model organism, although classical genetic screens appear to be saturated. The scientific challenge would be to design sensitive forward and reverse genetic screens that permit the isolation of novel genes involved in apoptosis.

1.3. Engulfment of Apoptotic Cell Corpses

The efficient removal of apoptotic cells is crucial for tissue homeostasis in multicellular organisms. Clearance of apoptotic cells prevents secondary necrosis and the subsequent release of potentially harmful intracellular contents into the surrounding tissue [67]. This is of special importance, as the failure to properly remove apoptotic cells has been implicated in the onset of autoimmune and inflammatory diseases in humans [68].

1.3.1. Cell Biological Properties

Engulfment or phagocytosis represents the culmination of the apoptotic program. In contrast to mammals, *C. elegans* does not have professional phagocytes. Instead, apoptotic cells are engulfed by neighbouring cells [69]. Engulfment by non-professional neighbours can also be observed in mammals in early development (prior to the generation of mature phagocytes) as well as in situations where professional macrophages are not present [70].

1.3.2. Recognition of Apoptotic Cells: 'Eat-me' Signals, Receptors and Opsonising Molecules

'eat-me' signals

In order to be recognised by phagocytes, apoptotic cells have to expose an 'eat-me' signal on their surface that marks them for engulfment. Among the surface changes on apoptotic cells, the most common and best characterised is the loss of phospholipid asymmetry in the plasma membrane and the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (Figure 4) [71,72]. Exposure of PS is an early event during the apoptotic process and appears to be a universal feature of programmed cell death [73,74]. However, the exact mechanism of PS exposure remains elusive. It has been postulated that PS exposure is mediated by activation of a scramblase, a bidirectional, Ca^{2+}-dependent, substrate

unspecific activity and by the inactivation of a translocase, an inwardly-directed, Ca^{2+}-sensitive PS specific activity; but in vivo evidence for the participation of these molecules in PS translocation is still missing [75,76]. Whether in *C. elegans* apoptotic cells expose PS as well has not been unequivocally demonstrated yet, but various data suggest that PS exposure on apoptotic cells is evolutionarily conserved (see *Chapter 4*) [72,74,77].

Receptors

In mammals, many receptors have been described that are able to bind PS on apoptotic cells, including scavenger receptors, such as CD36, CD68 and Lox1; certain integrins, such as the $\alpha_v\beta_3$ integrin receptor; several lectins; the receptor tyrosine kinase MER; the low-density lipoprotein related protein CD91; the LPS receptor CD14 and the PS receptor PSR [67,78-80]. Most of them do not discriminate between PS and other anionic phospholipids, but the phosphatidylserine receptor (PSR) binds PS in a stereospecific manner and is expressed on all cells that are able to engulf apoptotic cells [81]. In vivo evidence that PSR is involved in the engulfment of apoptotic cells comes from the observation that PSR knock-out mice accumulate apoptotic cells in the lung [82], liver and thymus [83]. However, $PSR^{-/-}$ mice die within 24 hours after birth by lung failure and exhibit defects in the developing brain as well as in erythroid and T-lymphoid cell differentiation, suggesting that beside cell corpse engulfment PSR plays a crucial role during development [82,83]. Novel data from Böse and colleagues (2004) demonstrate that PSR is essential for the development and differentiation of multiple organs during embryogenesis [84]. However, PSR knock-out mice, generated by Böse and colleagues, show no engulfment defect, indicating that PSR rather functions in embryonic organ development than apoptotic cell removal.

The *C. elegans* genome encodes a homologue of PSR and recently, a *psr-1* knock-out mutant has been generated by Wang et al. (2003). The authors claim that *psr-1* mutant worms are defective for engulfment and suggest that PSR-1 acts in the same engulfment pathway as CED-2, CED-5, CED-10 and CED-12, possibly through direct interaction with CED-5 and CED-12 [85]. However, these findings are still under debate: firstly, PSR

contains multiple nuclear localisation signals that promote nuclear localisation of a GFP fusion protein [86], suggesting that PSR rather plays a role in the nucleus than at the cell surface; secondly, in my hands, both deletion mutants *psr-1(tm469)* and *psr-1(ok714)* do not show any engulfment defect (Züllig and Hengartner, *Chapter 3*). Clearly, the role of PSR-1/PSR needs to be examined further and subcellular localisation studies might provide new insights into its in vivo function.

Opsonising Molecules

In mammals, several proteins have been shown to act as bridging molecules that can bind to 'eat-me' signals on the surface of apoptotic cells and link them to receptors on the phagocyte [79,80,87]. Annexin I, an intracellular protein that translocates from the cytosol to the outer leaflet of the plasma membrane during apoptosis [88,89] has been proposed to be the bridging molecule for PS and PSR. Annexin I is normally a cytosolic protein, but has been reported to be secreted into the extracellular space upon induction of apoptosis [88]. Secreted Annexin I binds to the surface of the apoptotic cell, thereby promoting its efficient engulfment by bystander phagocytes. RNAi mediated knockdown of the *C. elegans* orthologue *nex-1* also causes a defect in the clearance of apoptotic cells in worms, suggesting that the role of Annexin I in engulfment is evolutionarily conserved [88]. Surprisingly, a candidate *C. elegans nex-1* mutant shows no obvious engulfment defect (Neukomm and Hengartner, *unpublished*). However, further work will be required to resolve this discrepancy.

Among the known opsonising molecules in phagocytosis, the milk fat globule epidermal growth factor (EGF) factor 8 (MFG-E8) is the best characterised. MFG-E8 has been shown to promote engulfment of apoptotic cells by binding exposed PS on the apoptotic cell and the $\alpha_v\beta_3/\alpha_v\beta_5$ integrins on the phagocyte [90,91]. MFG-E8 knock-out mice are viable, but their tingible body macrophages fail to efficiently engulf apoptotic B cells. Notably, binding of tingible body macrophages to apoptotic B cells is not impaired in mice lacking MFG-E8, suggesting that MFG-E8 is required for internalisation rather than for recognition and binding of apoptotic cells [92,93]. Interestingly, MFG -

E8$^{-/-}$ mice acquire an age-dependent autoimmune disorder, a disease often associated with impaired clearance of apoptotic cells. Although a potential homologue of MFG-E8 exists in *C. elegans*, it does not appear to be involved in the engulfment of apoptotic cells (Charette and Hengartner, *unpublished*).

1.3.3 Genes Involved in the Engulfment of Apoptotic Cells in *C. elegans*

In *C. elegans*, apoptotic cells are recognised and rapidly engulfed by neighbouring cells [23]. So far, seven genes – *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced–7*, *ced-10* and *ced-12* – have been identified that are required for proper engulfment of apoptotic cells [20-22]. However, none of these known genes is absolutely essential for the engulfment process, as many dying cells are still properly removed in mutant animals. Double mutant analyses grouped the engulfment genes into two distinct, partially redundant pathways: *ced-1*, *ced–6*, and *ced-7* in one and *ced-2*, *ced-5*, *ced-10* and *ced-12* in the other [21,94]. However, recent studies suggest that the two pathways converge at the level of *ced-10* (Kinchen et al., *unpublished*) (Figure 5).

ced-1, ced-6 and *ced-7*

The first group of known engulfment genes in *C. elegans* consists of *ced-1*, *ced-6* and *ced-7.* Mutations within this group cause a defect in engulfment of apoptotic cells, but despite the engulfment defect, mutant worms are healthy and their morphology is overtly normal (Figure 6). *ced-1* encodes a transmembrane protein that consists of an amino-terminal extracellular domain, which contains 16 motifs of an atypical EGF-like repeat, a single transmembrane domain and a carboxy-terminal intracellular domain containing a NPXY and a YXXL motif. These motifs are present in the cytoplasmic tail of many membrane receptors and have been shown to mediate interactions with cytoplasmic adaptor proteins [95]. The precise mammalian homologue of CED-1 is still under debate: The mammalian proteins, MEGF-6 (*m*ultiple *e*pidermal *g*rowth *f*actor domain-like protein) and SREC (*s*cavenger *r*eceptor from *e*ndothelial *c*ells), identified as most similar to CED-1 [96], fail to show any homology with the cytoplasmic region of CED-1

and do not contain the NPXY or YXXL motifs. Database searches based on the presence of the EGF-like and NPXY motifs, identified CD91/LRP (*l*ow density lipoprotein receptor-*r*elated *p*rotein) as a potential homologue of CED–1 [97]. CD91/LRP has been shown to interact with Gulp, the mammalian homologue of CED-6, suggesting that CD91/LRP is the functional homologue of CED-1. However, a recent publication by Callebaut et al. (2003) describes the existence of an EMI domain within the N-terminus of CED-1 [98]. The EMI domain is a small module rich in cysteines and has been found in EMILINS and multimerin, which are glycoproteins of the extracellular matrix. In this study, the human protein MEGF-10, which also contains a NPXY motif in its C-terminus, has been proposed as the orthologue of CED-1.

By expressing a functional CED-1::GFP fusion protein, Zhou et al. (2001) showed that CED-1 localises to cell membranes and clusters around neighbouring cell corpses [96]. These observations support the idea that *ced-1* encodes a cell surface receptor necessary for phagocytosis. However, the ligand that binds CED-1 remains elusive. In vitro binding studies done by Su et al. (2002) show that the NPXY motif in the cytoplasmic tail of CED-1 physically interacts with the PTB (*p*hospho*t*yrosine *b*inding) domain of CED-6 [97]. These data provide evidence that CED-1 acts through the adaptor protein CED-6 to propagate the engulfment signal to the cell interior.

ced-6 encodes an adaptor protein, consisting of a PTB domain at its amino-terminus, a central leucine zipper domain that mediates homodimerisation, and a proline-rich region in its carboxy-terminal half that contains several potential SH3-binding sites [99,100]. Overexpression of CED-6 significantly suppresses the engulfment defect caused by putative null mutations of *ced-1* and *ced-7*, indicating that *ced-6* might act downstream of *ced-1* and *ced-7*. Thus, it is likely that CED-6 is part of a signalling cascade that promotes recognition and engulfment of apoptotic cells in *C. elegans*. A physical link between the PTB domain of CED-6 and the NPXY motif in the cytoplasmic domain of CED-1 found by Su et al. (2002) supports the idea of an engulfment signalling cascade where CED-6 functions as an adaptor molecule. This signal transduction pathway might be conserved from *C*.

elegans to humans as overexpression of hCED-6/Gulp (eng*ul*fment ada*p*ter protein) partially rescues the engulfment defect of *ced-6* mutant worms [101] and specifically promotes the phagocytosis of apoptotic cells [102]. Gulp interacts with CED-1 and CD91/LRP, providing further evidence for the evolutionarily conservation of CED-6 and hCED-6/Gulp [97]. The only known downstream target of CED-6 is CED-10, which, when overexpressed, is able to partially rescue the engulfment defect of *ced-6* mutant worms (Kinchen et al., *unpublished*). How CED-6 is linked to CED-10 remains to be determined.

C. elegans CED-7 is a member of the ABC (*A*TP-*b*inding *c*assette) transporter family with most similarity to mammalian ABCA1 [103]. Like other members of the ABC transporter superfamily, CED-7 consists of two similar halves that contain a hydrophobic region with six putative transmembrane domains and a predicted ATP *n*ucleotide-*b*inding *d*omain (NBD). A unique *h*ighly *h*ydrophobic domain (HH1) is localised between the two halves of the protein. However, the functional or structural significance of this domain remains to be determined. ABCA1 is highly expressed on macrophages engaged in the engulfment and clearance of dying cells, supporting its role in the recognition and removal of apoptotic cells [104]. ABC transporters are known to mediate the transport of a variety of substrates, including ions, sugars, vitamins, phospholipids, peptides and even proteins. Importantly, ABCA1 plays also a crucial role in cholesterol metabolism, as in humans loss of ABCA1 function results in Tangier disease, an autosomal recessive disorder of lipid metabolism characterised by a defect in the efflux of cholesterol [105].

C. elegans CED-7 is widely expressed in embryos and localises to the plasma membrane, consistent with its role in the engulfment of embryonic cell corpses [103]. Most importantly, whereas all engulfment genes described so far are clearly required only in the engulfing cell to promote the recognition and removal of apoptotic cells, *ced-7* function is also required in dying cells [103]. This finding raises the possibility that *ced-7* might have different functions in apoptotic and engulfing cells. Interestingly, Zhou et al. (2001) have shown that *ced-7* function is required for CED-1 to cluster around cell corpses, as CED–1::GFP clustering is greatly diminished in *ced-7* mutants [96]. But

CED–1::GFP is still localised to cell surfaces, indicating that mutation of *ced-7* does not affect the membrane localisation of CED-1. Why CED-7 is important for CED-1 clustering is currently an open question.

ced-2, ced-5, ced-10, and *ced-12*

The second group of engulfment genes, which includes *ced-2*, *ced-5*, *ced-10*, and *ced-12*, is better understood. *ced-2*, *ced-5*, *ced-10*, and *ced-12* are members of a Rac GTPase signalling pathway and encode homologues of the mammalian proteins CrkII, Dock180, Rac1 and Elmo, respectively. In *C. elegans* mutations in any of these genes lead to defects in phagocytosis and in distal tip cell (DTC) migration [21,22,103,106-108].

The most upstream component of this pathway is CED-2/CrkII. Originally, CrkII was identified as the cellular homologue of a viral oncogene, v-Crk, in chicken tumour samples [109]. It has since been implicated in a variety of cell shape and cytoskeletal changes [110]. CrkII encodes an adaptor protein, consisting of an SH2 (Src-homology-2) domain followed by two carboxy-terminal SH3 (Src-homology-3) domains [111]. CrkII localises to focal adhesions with $p130^{Cas}$ (*C*rk-*a*ssociated *s*ubstrate) and Dock180, a protein similar to CED-5 that was isolated on the basis of its physical interaction with CrkII [103,112]. In *C. elegans* CED-2 and CED-5 also physically interact [108]. But how CrkII/CED-2 exactly functions, remains unclear. Albert and colleagues (2000) proposed a model in which CrkII binds to the $\alpha_v\beta_5$ integrin receptor and recruits the $p130^{Cas}$-Dock180 complex to the membrane, thus allowing Rac1 activation and rearrangement of the cytoskeleton [113]. However, there is no clear $p130^{cas}$ homologue in worms, and integrins do not seem to play any evident role in phagocytosis [114], although they do affect the adhesion and motility of many cells [115].

ced-5 encodes a protein similar to Dock180 and its function is required for cell corpse removal and DTC migration [22,103]. Expression of human Dock180 in *C. elegans* rescues the DTC migration defect of *ced-5* mutants, suggesting that the function of CED-5 and Dock180 is evolutionarily conserved [103]. CED-5/Dock180 interacts with CED-2/CrkII and CED-12/Elmo,

indicating that these three proteins form a ternary complex, where Dock180 serves as a bridging molecule for CrkII and Elmo [21,108,112]. This ternary complex is required for intracellular signal transduction, resulting in the cytoskeletal rearrangements during both cell migration and the engulfment of apoptotic cells. The Docker domain (aa1111-aa1657) of Dock180 has been shown to interact specifically with nucleotide free Rac [116]. Although Dock180 does not contain a conventional catalytic domain for guanine nucleotide exchange on Rac, it has been shown that Dock180 and Elmo together mediate GTP loading of Rac, revealing an unconventional Rac-GEF activity through the Dock180-Elmo complex [116]. Importantly, novel data suggest that Elmo directly interacts with the small GTPase RhoG in a GTP dependent manner to induce Rac1 activation [117]. The authors further demonstrate that Dock180, Elmo and RhoG form a ternary complex, which translocates to the plasma membrane and activates Rac1, leading to integrin-mediated cell spreading and axon outgrowth. Surprisingly, recent biochemical studies showed that a large fraction of the Dock180/Elmo complex localises to the nucleus, where it displays robust GEF activity [118]. The biological significance of the nuclear localisation of the Dock180/Elmo complex remains to be determined.

CED-12 acts in the engulfment pathway together with CED-2, CED-5, and CED-10 to promote apoptotic cell corpse removal and DTC migration [21,106,107]. *ced-12* encodes a protein with a pleckstrin homology (PH) domain and an SH3 binding motif. The molecular function of CED-12/Elmo is still unknown, as the protein has no significant homology to any other protein class. In vitro binding studies showed that CED-12/Elmo binds directly to CED-5/Dock180 via a PXXP motif in its C-terminus and to RhoG via its N-terminus; this interaction has been proposed to promote Rac GDP/GTP exchange, leading to cytoskeletal rearrangements required for phagocytosis and cell motility [21,117]. The function of *C. elegans* CED-12 and mammalian Elmo is evolutionarily conserved, as overexpression of Elmo rescues the DTC migration defect in *ced-12* mutant worms [21]. However, the engulfment defect caused by *ced-12* loss-of-function cannot be rescued by Elmo overexpression (noteworthy, this holds also true for Dock180 overexpression in *ced-5* mutant

worms), suggesting that the ability to function in engulfment and long-term cell migrations has diverged through evolution [21,103]. Importantly, this hypothesis has been supported by the finding that Dock4, a member of the CDM gene family encoding regulators of small GTPases, rescues the engulfment, but not the DTC migration defect of *ced-5* mutants [119], indicating that Dock4 and Dock180 together reconstitute the functional properties of CED-5.

C. elegans ced-10 encodes a protein similar to Rac1. CED-10/Rac1 is a small GTPase, which belongs to a subgroup of the Ras-GTPase superfamily involved in the control of cytoskeletal organisation and cell extensions [108,120]. GTPases act as a molecular switch: they cycle between an inactive GDP-bound state and an active GTP-bound state. GTPase activating proteins (GAPs) facilitate GTPase activity leading to the inactive state, whereas GTP exchange factors (GEFs) stimulate the exchange of GTP for GDP, leading to the active state [108,121,122]. The *C. elegans* genome encodes three Rac-like genes, *ced-10*, *mig-2*, and *rac-2* [108,122]. However, only *ced-10* is required for cell corpse removal, whereas *mig-2* and *rac-2* play a role in axon pathfinding and various cell migrations [123].

CED-10 has been shown to be involved in DTC migration and the phagocytosis of apoptotic cells [108]. Overexpression of *ced-10* can compensate for the engulfment defect in *ced-2*, *ced-5*, and *ced-12* mutants, implicating that *ced-10* functions downstream of *ced-2*, *ced-5*, and *ced-12* [21,108]. However, recent data demonstrate that the engulfment defect caused by mutations in *ced-1*, *ced-6*, or *ced-7* can also be partially rescued by *ced-10* overexpression, suggesting that *ced-10* not only functions downstream of *ced–2*, *ced-5*, and *ced-12*, but also downstream of (or in parallel to) *ced-1*, *ced-6*, and *ced-7* in the engulfment of apoptotic cells (Kinchen et al., *unpublished*). Notably, double mutants that interrupt both engulfment pathways, showed little or no reduction in the number of apoptotic cell corpses following induction of *ced-10* overexpression, suggesting that rescue of the engulfment defect in single mutants is mediated by overactivation of the parallel pathway.

In mammalian cells, Dock180 and Elmo function as a bipartite Rac-GEF, promoting engulfment and cell migration by mediating Rac1 activation [116,124]. Recent studies suggest that the small GTPase RhoG interacts with Elmo and forms a ternary complex with Dock180 to induce Rac1 activation, important for integrin-mediated cell spreading and neurite outgrowth [117]. RhoG itself has been shown to be a target of the mammalian GEF Trio [125]. In *C. elegans*, it has been assumed that CED-5 and CED-12 function in a similar way to activate CED-10; however, *mig-2*, the *C. elegans* orthologue of RhoG only plays a role in cell migration and axon pathfinding, but not in engulfment of apoptotic cell corpses [123,126]. Confirmatively, UNC-73, the *C. elegans* homologue of Trio activates MIG-2 and is only required for cell migrations and axon guidance, but not for cell corpse removal [123,126].

Effectors, which function downstream of CED-10/Rac1 in the engulfment of apoptotic cells in *C. elegans* have not been identified yet, though several have been shown to be involved in cell migration during morphogenesis, acting in a CED-10/Rac1 dependent manner [127,128].

1.3.4. Engulfment Promotes Programmed Cell Death

It has long been believed that caspase activation represents a point of no return in the process of programmed cell death. However, this view has dramatically changed with the observation that blocking engulfment enhances cell survival when cell death is weakly impaired [129,130]. Thus, genes that mediate cell corpse removal can also function to actively kill cells. But how does the engulfment machinery contribute to cell killing? One can imagine that activated CED-3 cleaves as yet unknown substrates, leading to exposure of an ‘eat-me’ signal, which engages the apoptotic machinery. The so-called ‘back-up plan’ model implies that exposure of an ‘eat-me’ signal by weak CED-3 activation is not sufficient to kill the cell. Rather, exposure of the ‘eat-me’ signal on the surface of the apoptotic cell allows its recognition and proper removal by the engulfing cell. Alternatively, the so-called ‘positive-feedback’ model proposes that doomed cells signal to neighbouring cells their

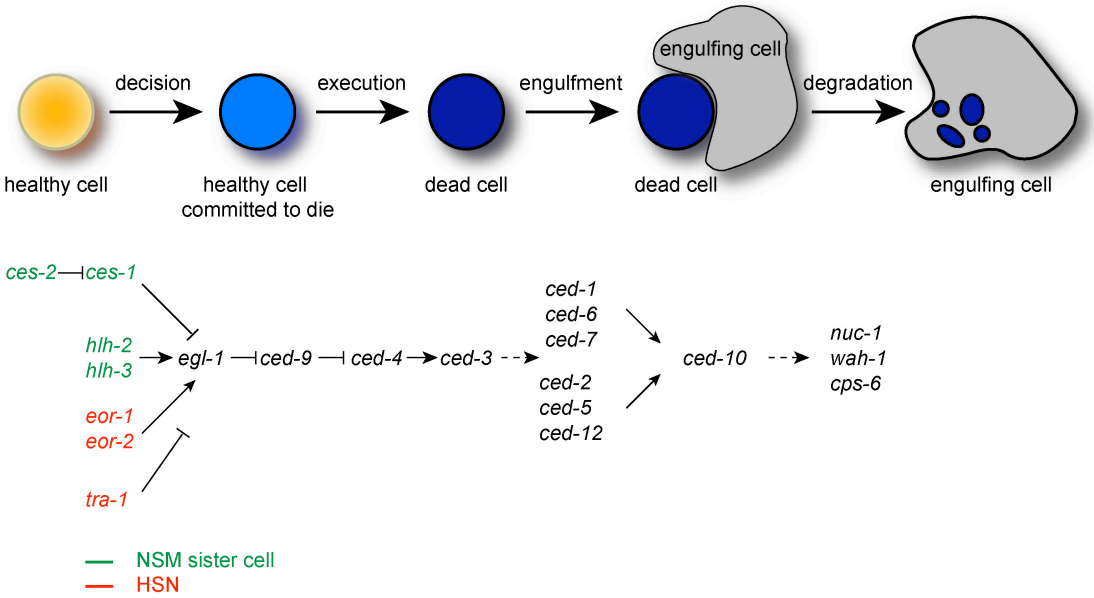
readiness to die, whereupon the engulfing cell generates a positive feedback signal that goads the doomed cell to final death.

1.3.5. Future Directions

Although our knowledge about engulfment has amazingly increased since the discovery of the first engulfment genes in 1983 [20], it is apparent that many components of the engulfment machinery have not been discovered yet. Especially, the signal(s) that mark the apoptotic cell for engulfment remain elusive. Furthermore, the downstream targets of the phagocytic machinery that lead to cytoskeletal rearrangements of the actin network necessary for proper engulfment of the apoptotic cell are still unknown. In contrast to mammals, *C. elegans* provides the opportunity to study engulfment in a simple model organism, bypassing the difficulties of studying a sophisticated process in a complex mammalian organism.

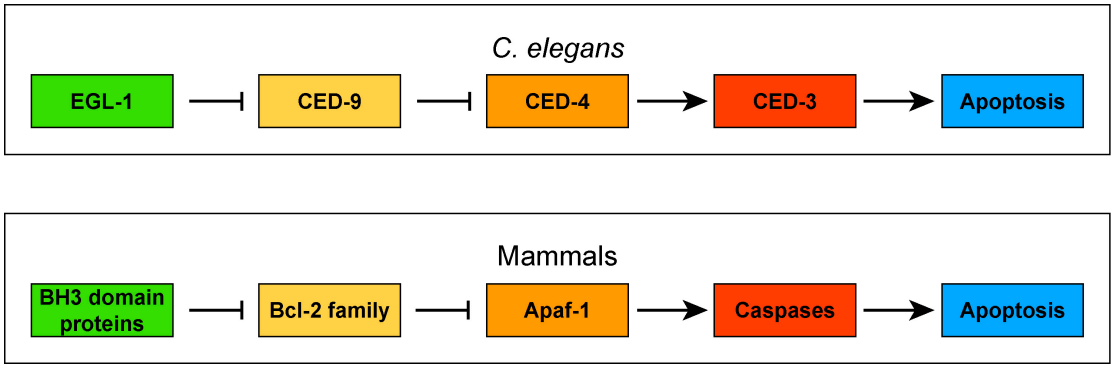
1.4. Figures and Tables

Figure 1: The Genetic Pathway of Programmed Cell Death in *C. elegans*



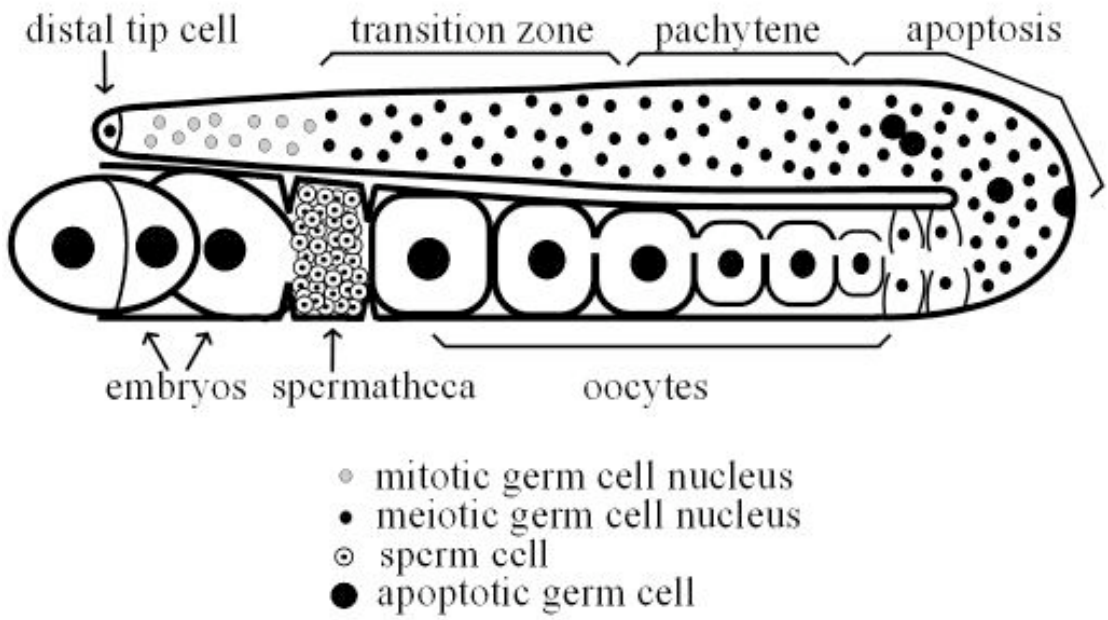
The genetic pathway of programmed cell death in *C. elegans* has been divided into four, genetically separable steps: (I) decision to die, (II) death execution, (III) engulfment, and (IV) DNA degradation. Genes involved in the decision whether to live or to die act only in a few discrete cells; in contrast, genes functioning in the last three steps virtually affect all known programmed cell deaths. The core apoptotic machinery, consisting of *ced-9*, *ced-4*, and *ced-3*, is essential for all programmed cell deaths. The seven engulfment genes – *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10,* and *ced-12* – have been grouped into two partially redundant pathways that converge at the level of *ced-10*. Defects in any of the engulfment genes affect apoptotic cell corpses removal, causing a persistent cell corpse phenotype. The final phase of the apoptotic program comprises the degradation of the apoptotic cell inside the engulfing cell.

Figure 2: The Genetic Pathway of Programmed Cell Death Is Conserved Through Evolution



The key molecules of programmed cell death are conserved between *C. elegans* and mammals. The core apoptotic machinery is activated when EGL–1, a pro-apoptotic BH3-only Bcl-2 family member, binds CED-9 and displaces CED-4. CED-9 is an anti-apoptotic member of the Bcl-2 family of proteins that exerts its inhibitory function by binding the pro-apoptotic protein CED-4. Released CED-4 (*C. elegans* homologue of mammalian Apaf-1) undergoes oligomerisation, thereby promoting autoactivation of the caspase CED-3 by an induced proximity mechanism. Activated CED-3 cleaves downstream targets leading to apoptotic cell death.

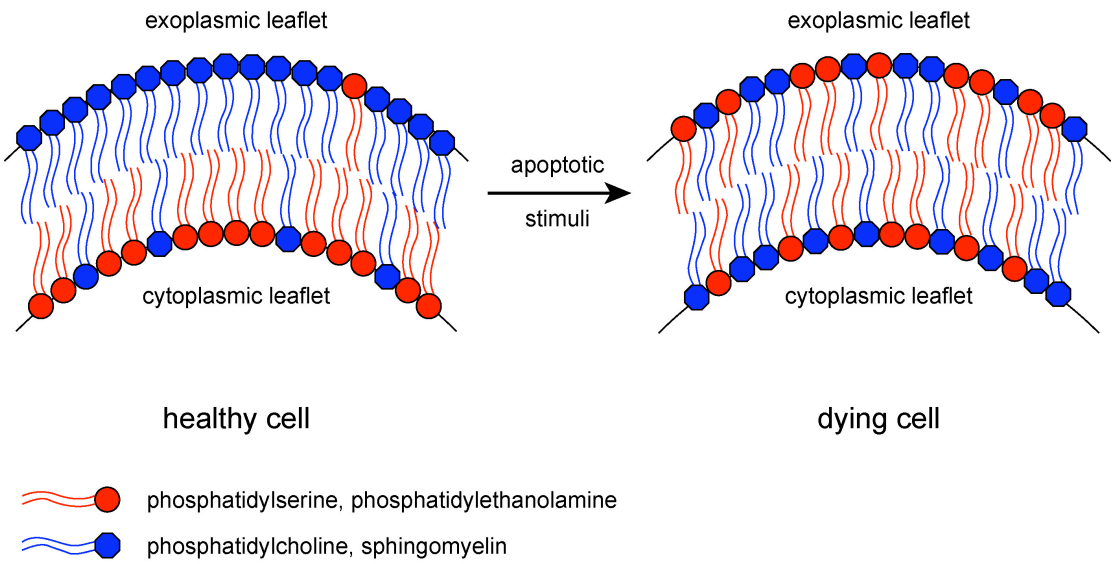
Figure 3: Structure of the Adult *C. elegans* Hermaphrodite Gonad



Courtesy of Guillaume Lettre

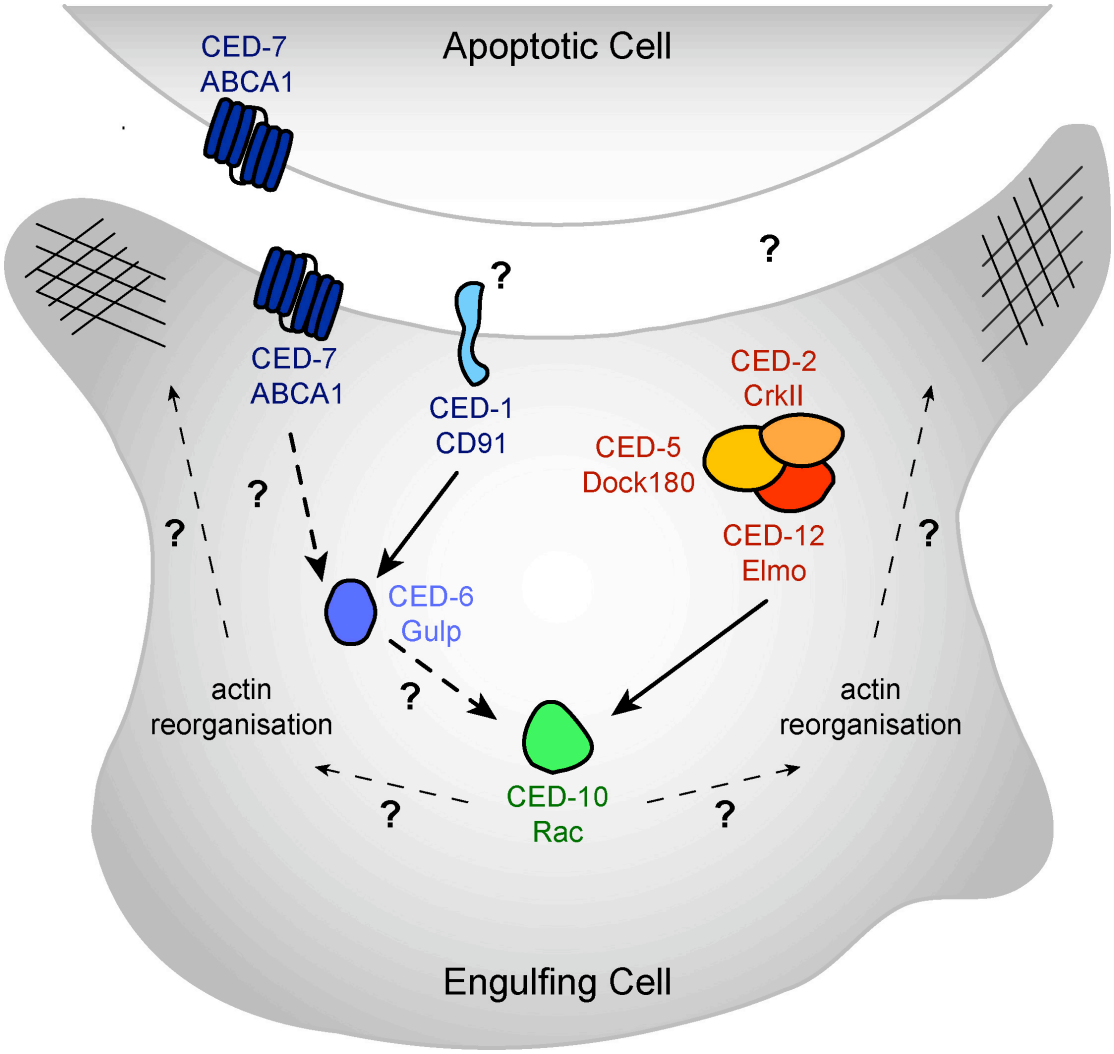
The hermaphrodite gonad consists of two U-shaped tubular arms that are connected at a common uterus. Each gonad has a distal-to-proximal polarity with the vulva defining the proximal opening of the gonad. At the distal end of each gonad arm, the somatic distal tip cell (DTC) provides a signal that is required for mitotic cell proliferation. Germ cells in the range of 1 to ~20 cell diameters from the DTC proliferate and serve as a stem-cell population. Moving proximally, germ cells exit mitosis and enter meiosis I (transition zone). When cells exit from the pachytene stage, they become sensitive to undergo apoptosis. Apoptotic germ cells cellularise from the common syncytium and are swiftly engulfed by the somatic sheath cells, which surround the gonad. Surviving germ cells progress around the loop and through the proximal gonad, where they become mature oocytes. Ovulated oocytes are fertilised in the spermatheca and accumulate in the uterus till they are laid through the vulva.

Figure 4: The Phospholipid Asymmetry of the Plasma Membrane Is Lost in Apoptotic Cells



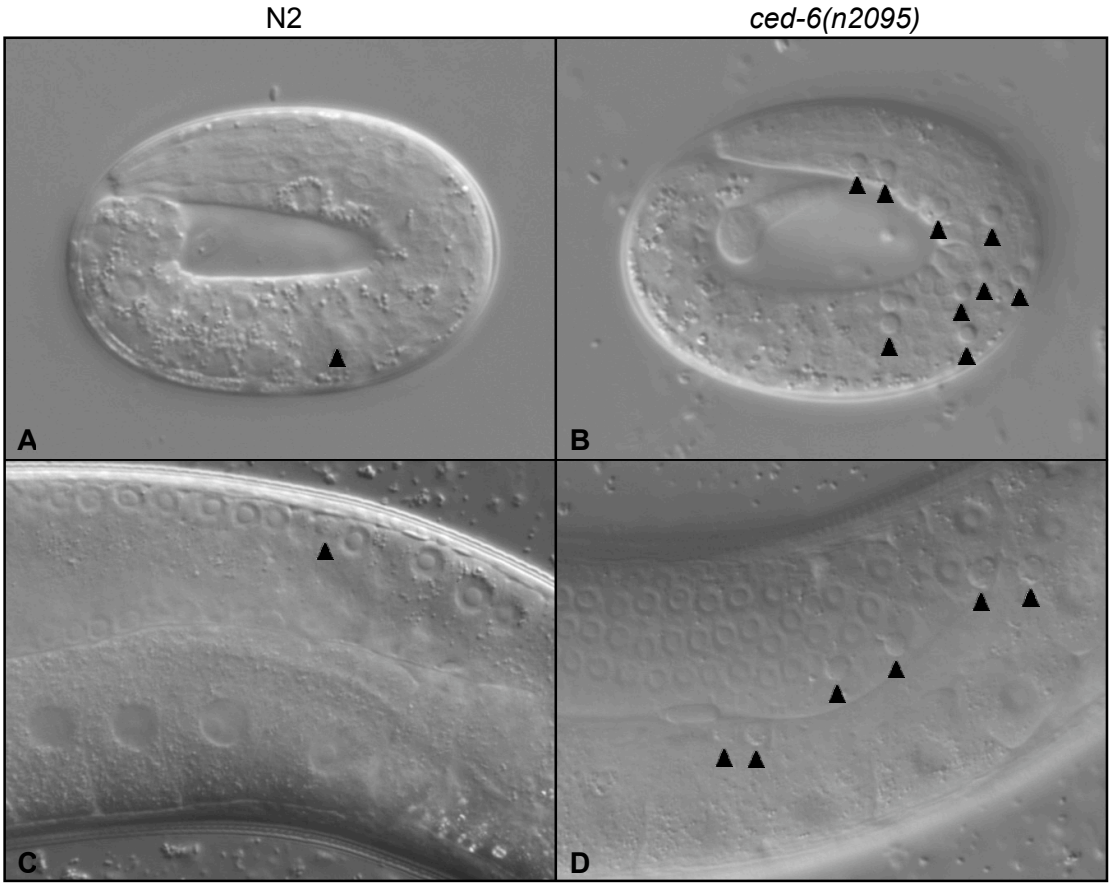
In cells undergoing programmed cell death the asymmetric distribution of the phospholipids in the plasma membrane is lost and PS is translocated from the inner to the outer leaflet of the plasma membrane.

Figure 5: Two Partially Redundant Pathways Mediate Engulfment of Apoptotic Cells in *C. elegans*



In *C. elegans* seven genes – *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, and *ced-12* – have been identified that are required for efficient engulfment of apoptotic cells. Genetic analyses grouped the engulfment genes into two distinct, partially redundant pathways: *ced-1*, *ced-6*, and *ced-7* in one, and *ced-2*, *ced-5*, *ced-10*, and *ced-12* in the other. Interestingly, novel results suggest that these two pathways converge at the level of *ced-10*. However, little is known about the molecules that mark the apoptotic cell for engulfment or about downstream effectors of *ced-10* that promote the rearrangement of the cytoskeleton (indicated by question marks in the figure).

Figure 6: Mutations in the Engulfment Genes Result in Persistent Cell Corpses



Apoptotic cell corpses can be observed as highly refractile disks by Nomarski microscopy. In wild-type worms apoptotic cell corpses are recognized and swiftly engulfed by an adjacent cell. On average, zero to one apoptotic cell corpse can be detected in the head region of a late 3-fold wild-type embryo (A); in embryos mutant for any one of the engulfment genes, e.g. *ced-6 (n2095)*, many apoptotic cell corpses (arrow heads) persist (B). In the hermaphrodite germ line more than half of all generated germ cells die by undergoing apoptosis. In wild-type hermaphrodites apoptotic germ cells are swiftly engulfed by the gonadal sheath cells, resulting in only two to three visible apoptotic germ cells per gonad (C). In contrast, in the germ line of an engulfment defective hermaphrodite, e.g. *ced-6(2095)*, many apoptotic germ cells fail to be engulfed and persist in the gonad for many hours or even days (D).

Table 1: Factors Involved in Apoptotic Cell Corpse Engulfment

Mammals	*C. elegans*	Description
Receptor Molecules		
ABCA1	CED-7	ATP binding cassette transporter
CD91/LRP receptor	CED-1	calreticulin/heat shock protein CD36,
CD68, LOX-1, SR-A	CED-1	scavenger receptors
(PSR)	(PSR-1)	phosphatidylserine receptor
Mer	—	receptor tyrosine kinase
β_2GPI receptor	—	β_2-glycoprotein-I receptor
CD14	—	lipopolysaccharide receptor
C3/C4	—	complement receptor 3/4
vitronectin receptor	—	integrin receptor
Opsonising Molecules		
AnxI	NEX-1	Annexin I
MFG-E8	—	milk-fat-globule-EGF-factor 8
Gas6	—	growth arrest-specific 6
β_2GPI	—	β_2- glycoprotein-I
Prot-S	—	protein S
MBL	—	mannose-binding lectin
SP-A/D	—	lung surfactant protein A or D
C1q	—	complement protein
C3b/bi	—	complement protein
Signalling Molecules		
CrkII	CED-2	SH2/SH3 adaptor protein
Dock180	CED-5	guanine nucleotide exchange factor
Gulp	CED-6	PTB adaptor protein
Ras	CED-10	small GTPase
Elmo	CED-12	GEF co-factor

Table 1 lists all molecules known to play a role in the removal of apoptotic cells in mammals and *C. elegans*. The function of PSR in apoptotic cell corpse engulfment is still under debate.

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Chapter 2

Tickling Macrophages, a Serious Business

2.1. Preface

This perspective was published in *Science* **304**, 1123-4 (2004) and discusses a paper from Hanayama et al. (2004) that was published in the same issue. I included this perspective in my thesis as it deals with the phagocytosis of apoptotic cells and might give an understanding of the function of the bridging molecule MFG-E8, which binds PS on apoptotic cells and stimulates their phagocytic uptake.

variety of *vir* mutants, they established a temporal order of proteins the DNA comes in contact with as it journeys through the T4S apparatus. In so doing, they refined the model of bacterial DNA transfer in unprecedented detail.

The beauty of the TrIP assay lies in its simplicity and its sensitivity. In this procedure, the authors treated *Agrobacterium* cells with formaldehyde to cross-link the DNA and interacting proteins. They then used specific antibodies to the various T4S components to immunoprecipitate DNA-protein complexes from the cross-linked *Agrobacterium* extracts. Finally, they assessed coprecipitation of the T-DNA using both standard and quantitative polymerase chain reaction (PCR). Because PCR requires only a miniscule amount of material, the TrIP assay is exponentially more sensitive than are more traditional assays for DNA-protein interactions. This remarkable sensitivity makes the assay ideal for exploring such interactions in vivo. Also, whereas standard genetic analyses typically allow only a positive or negative response to the question of whether a component of a multiprotein complex is essential for its function, quantitative TrIP distinguishes their relative contributions. TrIP analysis of the *vir* deletion mutants of *Agrobacterium* additionally provided detailed snapshots of the progress of the T-DNA through the translocation apparatus. Upon removal of each Vir protein, the remaining proteins were analyzed by TrIP for their association with T-DNA. This revealed exactly where in the apparatus the T-DNA was stalled. Documentation of these translocation intermediates provides what is unquestionably one of the most detailed models of extracellular secretion.

Although the conjugation machineries comprise the largest subfamily of T4S systems, many important pathogens use the T4S apparatus primarily for delivery of proteins rather than nucleic acids (*7*–*9*). *Helicobacter pylori*, for example, uses a T4S apparatus to inject virulence factors into the epithelial cells of the stomach lining, which may cause peptic ulcers and cancerous lesions. *Legionella pneumophila*, the causative agent of Legionnaire's disease, is an intracellular pathogen that employs a T4S system to evade the immune system upon uptake by macrophages. *Bordetella pertussis* secretes the pertussis toxin (that causes whooping cough) into the extracellular environment through the T4S system. The discoveries of Cascales and Christie regarding DNA transfer will very likely elucidate the mechanisms of type IV–mediated protein secretion in these human pathogens. Indeed, given that recognition of proteins is critical in the initiation of nucleic acid translocation, the DNA may be merely a "hitchhiker" in the proteins' journey across the bacterial envelope. Armed with new information about the architecture and function of these molecular transport systems (*10*), the medical and scientific communities are one step closer to hindering the virulence mechanisms of these pathogens. These transport pathways are attractive targets for the development of new therapeutic agents, which could block the secretion of virulence factors or perhaps thwart the spread of antibiotic resistance. Alternatively, we may be able to design a new class of antibiotics that opens the tightly regulated T4S channels from the outside, effectively compromising the integrity of the protective outer membranes. Potentially, we can turn what are invaluable modes of survival for a bacterium into its Achilles' heel. Another very exciting prospect is that the T4S systems of human pathogens may be engineered to courier designer DNA to specific cell types for gene therapy. Cascales and Christie have brought us a step closer toward making such possibilities a reality.

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CELL BIOLOGY

Tickling Macrophages, a Serious Business

Stephanie Züllig and Michael O. Hengartner

Cells undergoing programmed suicide (apoptosis) mark themselves for removal by presenting "eat-me" signals at the cell surface. The best known of these signals is undoubtedly the phospholipid phosphatidylserine (PS). Exposed PS is recognized by a variety of soluble "bridging" proteins, which themselves are bound by PS receptors expressed by phagocytic cells such as macrophages (see the figure). On page 1147 of this issue, Hanayama, Nagata, and colleagues (*1*) show that one of these bridging molecules, MFG-E8, is crucial for promoting the removal of apoptotic cells by specialized macrophages in secondary lymphoid organs. In addition, they reveal that mice lacking MFG-E8 suffer from late-onset autoimmune disease, a defect often associated with impaired clearance of apoptotic cells.

Animals eliminate cells that are in excess or potentially dangerous through apoptosis, a highly regulated process. The molecular basis of apoptosis has been studied extensively, as defects in apoptosis have been implicated in many human diseases including cancer and neurodegeneration (*2*). How the organism kills rogue or extraneous cells is, however, only half the story—the other half is how to get rid of the cell corpses. Work from many quarters reveals the existence of a vast array of receptors on macrophages and other "would-be eater" cells that allow them to recognize and engulf apoptotic cells. These receptors recognize specific changes at the surface of apoptotic cells that mark these cells for removal. The best characterized plasma membrane alteration is the translocation of PS, a phospholipid normally sequestered on the inner leaflet of the plasma membrane, to the outer leaflet, where it becomes accessible to bridging proteins present in the extracellular space (*3*, *4*).

Recognition of exposed PS comes with a twist: Most receptors do not bind to the phospholipid directly, but rather via soluble bridging proteins that bind to both the signal on the apoptotic cell and the receptor on the engulfing cell (see the figure). Hanayama *et al*. (*1*) investigated the in vivo role of one of these bridging proteins MFG-E8 (milk fat globule epidermal growth factor protein 8). As its name suggests, MFG-E8 was originally discovered as a component of the plasma membrane surrounding fat droplets present in the milk of lactating mice (*5*). Biochemical studies have shown that MFG-E8 binds to PS via two clotting factor VIII–homology domains present in its carboxyl terminus. MFG-E8 also is able to bind to either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin re-

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ceptors expressed by phagocytic cells via a typical tripeptide (RGD) motif located within the second of its two EGF repeats (*6*, *7*).

The MFG-E8 bridging protein is not only expressed in mammary glands, however—MFG-E8 mRNAs have been found in brain, spleen, and lymph nodes. The finding in spleen and lymph nodes suggests that MFG-E8 may be important for the clearance of dying immune cells. Indeed, Hanayama *et al*. found that in both spleen and lymph nodes, MFG-E8 expression was restricted to "tingible body" macrophages—these are specialized phagocytic cells present in the germinal centers of lymphoid organs (*1*). Germinal centers are regions where B cells become activated by antigen, proliferate, mature, and start producing antibody. But there are dangers as well as opportunities for germinal center B cells: Activated B cells that fail to recognize an antigen, or have only low affinity for the antigen, undergo apoptosis and are swiftly removed by the tingible body macrophages.

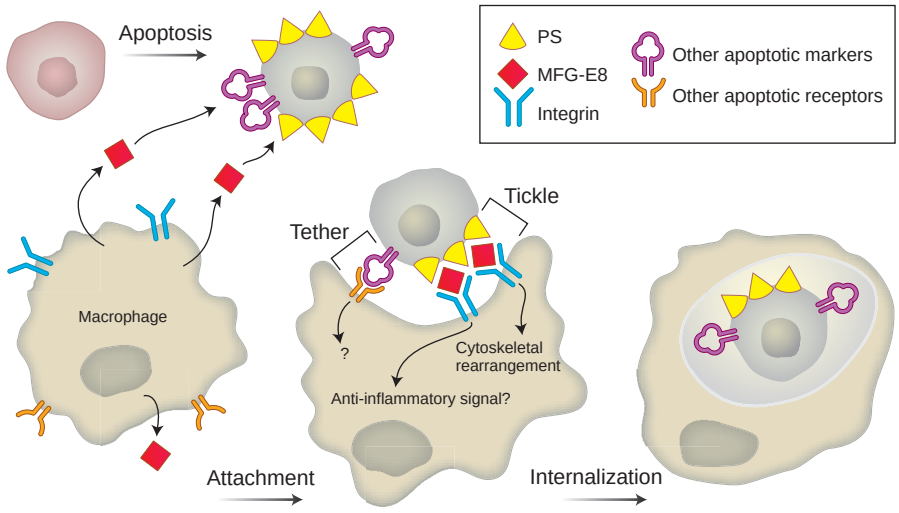
To address the connection between MFG-E8 and tingible body macrophages, Hanayama *et al*. generated mice lacking MFG-E8. These mice are viable but their tingible body macrophages fail to efficiently engulf apoptotic B cells (*1*). The investigators observed a similar engulfment defect in activated peritoneal macrophages (a second type of specialized phagocytic cell) from these mice. Using a clever trick, the authors could demonstrate that in both cases, absence of MFG-E8 blocked internalization of apoptotic cells but not their attachment to macrophages. In other words, the macrophages still effectively recognize apoptotic cells, but their desire or ability to swallow them is impaired.

One easy way to explain this observation is to posit that because of the multiplicity of eat-me signals and receptors, apoptotic cells still stick to macrophages but MFG-E8 is specifically required to initiate the subsequent cytoskeletal rearrangements needed for phagocytosis (see the figure). Such a two-step model of engulfment is termed "tether and tickle" (*4*, *8*). The Hanayama *et al*. study provides nice in vivo support for this model. Intriguingly, previous in vitro work suggested that the promotion of engulfment (the "tickle" step) is mediated by a PS receptor called PSR [although the recent observation that PSR is a nuclear protein (*9*) has raised questions regarding this functional assignment]. Perhaps PS exposure on the surface of apoptotic cells acts as a general "tickle" signal.

Mice lacking MFG-E8 develop two additional noteworthy phenotypes. First, they acquire an age-dependent autoimmune disease with excessive production of self-reactive antibodies. Development of autoimmunity may be the major negative consequence of impaired apoptotic cell clearance because unengulfed apoptotic cells undergo secondary necrosis, and necrotic cells—in contrast to apoptotic cells—activate the immune system (*10*). However, most mice lacking proteins needed for phagocytosis do not develop autoimmunity (apart from loss of C1q and the Mer receptor). What is particularly striking in the MFG-E8 mouse is that autoimmunity develops even though there appears to be extensive contact between apoptotic cells and macrophages. Previous studies suggested that binding of apoptotic cells to macrophages is sufficient to block macrophage activation (*11*). Perhaps the MFG-E8/integrin system is also required for this inhibitory signal.

The second notable phenotype of mice lacking MFG-E8 is a progressive splenomegaly. Interestingly, the distribution of cell types in the enlarged spleens of these mice is normal, suggesting a possible defect in immune cell turnover or physiology rather than a defect in differentiation. It will be interesting to identify the primary cause of this defect. Does MFG-E8 negatively regulate B cell proliferation independently of its role in cell corpse removal? Do persistent apoptotic cells result in an increased number of activated macrophages? Or does a cell clearance defect somehow interfere with the apoptotic removal of cells that should die, as seen in the nematode (*12*)?

What is the take home message of this story? MFG-E8 is also found in the brain and mammary gland. Could MFG-E8 also be involved in the removal of dying cells in ischemic brain tissue or in regressing mammary tissue after weaning? A recent report shows that Del-1, a homolog of MFG-E8, is expressed by macrophages isolated from thymus and fetal liver (*13*). Del-1 and MFG-E8 could conceivably promote removal of apoptotic cells in primary and secondary lymphoid organs, respectively, further broadening the pivotal role of this protein family in the removal of apoptotic cell corpses. Outside the immune system, other bridge/receptor pairs, such as annexin I/PSR, might contribute to the clearance of cell debris in other tissues. Clearly, tickling macrophages is a serious business and represents a research market that will continue to grow.



Tether and tickle. MFG-E8 is a bridging protein secreted by macrophages. Binding of MFG-E8 to apoptotic cells stimulates their removal by phagocytic cells. Engagement of the MFG-E8 receptor (possibly integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$) expressed by macrophages not only induces internalization of MFG-E8, but also may inhibit macrophage activation, thereby preventing the development of an autoimmune response against apoptotic cell epitopes. In the absence of MFG-E8 (not shown), apoptotic cells still bind to macrophages via other signals or receptors, but they cannot be engulfed.

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Chapter 3

Kinetic Analysis of Persistent Cell Corpse Removal in Engulfment-Defective Mutants of the Nematode *C.elegans*

3.1. Introduction

A common cell fate during *C. elegans* development is programmed cell death. Of the 1090 cells generated during hermaphrodite somatic development, 131 cells die by undergoing apoptosis [1-3]. Most (113/131) developmental cell deaths occur during embryogenesis, mainly between 250 and 450 minutes after fertilisation [3]. The remaining cell deaths fall into the second larval stage, following the divisions of several neuroblasts. However, after the second larval stage no more deaths are observed in the soma of hermaphrodites. Apoptotic cells in *C. elegans* are rapidly recognised and engulfed by neighbouring cells and are usually gone within less than an hour. Previous studies have identified at least seven genes – *ced-1*, *-2*, *-5*, *-6*, *-7*, *-10*, and -12 – that are required for efficient removal of apoptotic cell corpses in *C. elegans* [4-6]. Double mutant analyses grouped these engulfment genes into two distinct partially redundant groups that have been recently shown to converge at the level of *ced-10* (Kinchen et al., *unpublished)*. Importantly, mutation of any of these genes results in unengulfed cell corpses that persist for hours, or even days. However, most persistent cell corpses do eventually disappear, through an as yet unknown mechanism.

Of the known engulfment mutants, the function of *ced-7* is least understood. CED-7 is most similar to ABC (ATP-binding cassette) transporters that mediate the transport of a variety of substrates, including ions, sugars, vitamins, phospholipids, peptides, and proteins across extra- and intracellular membranes [7]. However, the mechanism by which ABC transporters achieve their substrate specificity is poorly understood. Like other eukaryotic members of the ABC transporter superfamily, CED-7 consists of two similar halves. Each half contains a hydrophobic region with six putative transmembrane domains and an ATP-binding domain, also known as the nucleotide-binding fold (NBF), consisting of the Walker A and B motives [8], and the ABC signature sequence, which is diagnostic for ABC transporters [7]. The two halves of the CED-7 protein are connected via a unique *h*ighly *h*ydrophobic (HH1) domain that is characteristic for all members of the ABCA1

subfamily [9]. Mouse ABCA1 has been shown to be expressed in macrophages engaged in engulfment and antibody-mediated steric blockade of ABCA1 severely diminishes the ability of macrophages to ingest apoptotic cells [10]. Interestingly, in mice lacking ABCA1, apoptotic cell corpses accumulate in the limb buds, confirming the involvement of ABCA1 in the clearance of apoptotic cells in vivo [11]. Forced expression of ABCA1 confers engulfment ability to non-phagocytic cells, demonstrating that ABCA1 is not only required but also sufficient for engulfment of apoptotic cells [11]. In addition to engulfment, ABCA1 promotes the efflux of cholesterol and the redistribution of PS across the plasma membrane [11]. The important role of ABCA1 in cholesterol efflux is mirrored in patients suffering from Tangier disease, a recessive disorder characterised by high levels of cholesterol esters in tissue macrophages [12].

Unlike the other engulfment genes, *ced-7* appears to be required specifically for the clearance of apoptotic cells during embryogenesis, but not during larval development, since most unengulfed corpses originally found in embryos rapidly disappear following hatching [13]. Interestingly, CED-7 is widely expressed in embryos, whereas in larvae and adults CED-7 expression is restricted to specific cells and the germ line [14]. CED-7 protein is localised to the plasma membrane, consistent with its sequence as an ABC transporter. Analysis of *ced-7* genetic mosaics showed that *ced-7* function is not only required in the engulfing but also in the dying cell [14]. This surprising observation suggests that *ced-7* activity might be important for the interaction between the cell surfaces of the dying and engulfing cells. However, it is not known whether *ced-7* might be required for the initial recognition of the dying by the engulfing cell, the subsequent adhesion between the dying and the engulfing cell, or even both.

Taken together, these results suggest that CED-7/ABCA1 plays an important role in the engulfment of apoptotic cells not only in worms but also in mammals. The specific characteristics of CED-7 make it an interesting studying object for further elucidation of the engulfment process.

Although the engulfment genes have been characterised genetically and molecularly, the persistent cell corpse phenotype has never been examined closely and the defects caused by the different engulfment mutants have not really been compared to each other. So far, it has not been known how long unengulfed cell corpses can persist in different engulfment mutants. Consequently, the final fate of unengulfed cell corpses has remained elusive too. Theoretically, unengulfed apoptotic cell corpses can undergo three distinct fates: I) they are never engulfed and persist forever, II) they are finally engulfed after a long persistence period, III) they undergo secondary necrosis. However, it is not known so far, which route persistent cell corpses might take. In this chapter, I present a detailed kinetic analysis of persistent cell corpse loss in the various engulfment mutant backgrounds. I show that persistent cell corpses have similar half-lives in all engulfment mutants except *ced-7*, suggesting that a common mechanism is responsible for persistent cell corpse disappearance in all but *ced-7* mutant backgrounds.

3.2. Results

3.2.1. A Kinetic Analysis of Cell Corpse Persistence

To learn more about cell corpse persistence in the various engulfment mutants, I decided to score the number of unengulfed cell corpses over time. I divided the embryonic and larval stages defined by Sulston et al. (1983) into 18 subclasses, which can be distinguished by Nomarski optics (Figure 1) [3]. Persistent cell corpses were scored in whole embryos at early developmental stages and in the head region from the early three fold stage onward in the wild type and in at least one allele (if possible the canonical one) of all the known engulfment genes. As published previously, apoptotic cell corpses are rapidly engulfed in the wild type, whereas in engulfment mutants, a significant number of cell corpses persist. Most engulfment mutants appear to have a maximum number of cell corpses at the two fold stage and are therefore quite similar. However, the decay curves differ significantly between *ced-7* and the other engulfment mutants: *ced-7* mutants show strikingly less persistent cell

corpses during larval development than the other engulfment mutants do (Figure 2). These data are consistent with previous studies, which showed that mutations in *ced-7* cause an engulfment defect during embryonic, but not during larval development [6]. Interestingly, all engulfment mutants, except *ced–7*, show unengulfed cell corpses even in the late L4 stage, demonstrating that many unengulfed cell corpses persist during larval development. However, unengulfed cell corpses do not persist forever, rather they are slowly removed over time (Figure 2).

3.2.2. Persistent Cell Corpses Are Not Removed Via the Classical Engulfment Pathway

Why do persistent cell corpses slowly disappear over time? Three simple models can be contemplated: I) the (defective) engulfment pathway recognises and removes persistent cell corpses, albeit inefficiently; II) a distinct 'salvage pathway' removes persistent apoptotic cell corpses; III) persistent apoptotic cell corpses undergo secondary necrosis and lyse.

The known engulfment mutants have originally been grouped into two distinct partially redundant pathways: *ced-1*, *-6*, *-7* in one and *ced-2*, *-5*, *-10*, and *-12* in the other [5,6]. Yet recent data suggest that both pathways converge at the level of *ced-10* (Kinchen et al., *unpublished*). The existence of two partially redundant engulfment pathways suggests that perturbing the function of just one pathway might not be sufficient to entirely abrogate the engulfment process. This partial redundancy might not only explain the relative high number of properly engulfed cell corpses in single engulfment mutants, but also the slow removal of persistent cell corpses during larval development. Indeed, previous experiments with conditional rescue constructs have shown that the classical engulfment machinery can promote the removal of both 'fresh' and persistent cell corpses [15,16].

To test this possibility, I scored unengulfed cell corpses in double mutants within one group (e.g. *ced-1; ced-7*) as well as in double mutants

between the two groups (e.g. *ced-7; ced-5*) and compared the number of persistent cell corpses (Figure 3). As it has been described before, double mutants within one group, show only a modest increase in engulfment defect, whereas double mutants between the two groups lead to a synergistic increase in the number of persistent cell corpses. This increase is particularly evident during larval development. However, as it is the case for single mutants, unengulfed cell corpses do not persist forever, not even in a strong *ced-7*; *ced-5* double mutant. Taken together, these findings strongly suggest that the classical engulfment pathway is not responsible for the loss of persistent cell corpses.

3.2.3. The Same Process Likely Mediates Persistent Cell Corpse Loss in All Engulfment Mutants Except *ced-7*

To further investigate the mechanisms of persistent cell corpse removal and to specially follow up the hypothesis of an alternative salvage pathway, I performed regression plot analysis of persistent cell corpses. Regression analysis fits a line between a set of data and estimates the relationship between the distinct variables. For this purpose, I transformed the persistent cell corpse numbers of single and double engulfment mutants into log numbers and generated regression plots using Statview 4.5 software. As almost all somatic cell deaths occur during embryogenesis, one can consider unengulfed cell corpses, which are still visible during larval development, as persistent cell corpses. Therefore, I decided to perform regression analysis of log corpses from the mid L1 larval stage onward. From the regression analysis one can learn the y intercept, i.e. the number of persistent cell corpses at the mid L1 time point – an indication of the strength of the engulfment defect - as well as the slope, from which one can calculate the half-life of persistent cell corpses (Table 1). Assuming that in a single engulfment mutant, persistent cell corpses can be eliminated through the other, partially redundant engulfment pathway, one would expect that elimination of the second pathway would greatly increase the persistence of unengulfed cell corpses. However, I found that persistent cell corpses showed

very similar decay curves (Figure 4) and half-lives (Table 1) in single and double engulfment mutants, providing further support that cell corpses in single engulfment mutants are not removed through the other engulfment pathway. Rather, I propose the existence of a 'salvage pathway', which stochastically removes persistent cell corpses even in a strong double mutant.

3.2.4. Efficient Removal of Persistent Cell Corpses in *ced-7* Mutants

Importantly, regression plot analysis of persistent cell corpses reinforces the distinct nature of *ced-7* mutants. Even though *ced-7* mutants show a strong engulfment defect in embryos, persistent cell corpses rapidly disappear during larval development, resulting in a significantly shorter half-life of cell corpses. It is possible that there is an activity, present in larvae but not in embryos, that is able to partially substitute for *ced-7* and allows the classical engulfment machinery to engulf persistent cell corpses. Alternatively, a distinct corpse removal mechanism, not present in the other engulfment mutants, allows a relatively efficient elimination of persistent cell corpses in *ced-7* mutants. Interestingly, the half-live of *ced-7* double mutants, e.g. *ced-1; ced-7* and *ced-7; ced-5* are both similar to either *ced-1* or *ced-5* single mutant.

3.2.5. A Genetic Screen for Genes That Act in Parallel to *ced-7*

In order to shed some light on the mechanisms that mediate removal of unengulfed cell corpses in *ced*-7 mutant larvae, I performed an ENU mutagenesis in a *ced-7* mutant background and screened for an enhancement of the engulfment defect during larval development. If a gene (or a pathway) acts in parallel to *ced*-7 to mediate cell corpse removal in larvae, then mutation of this gene in a *ced*-7 mutant background should lead to a strong engulfment defect, similar to that observed in the other engulfment mutants. Thus, I mutagenised *ced-7(n1996)* mutant hermaphrodites and screened the F3 animals for mutant larvae with an increased number of persistent cell corpses. I screened approximately 10'000 haploid genomes

and identified eight mutants with an increased engulfment defect. Mutations that bred true were backcrossed, mapped to chromosomes and tested for complementation with alleles of known engulfment genes. Unfortunately, all isolated mutations ended up being new alleles of engulfment genes that had already been identified. I found five new alleles of *ced-1*, two new alleles of *ced-2*, and one new allele of *ced-10* (Table 2).

Since there were only three alleles of *ced-2* known prior to our screen, I decided to sequence the two new alleles of *ced-2*. *op321* has a T to G transversion at position 298 in exon 1, changing His to Gln at aa98 and *op327* has a T to G transversion at position 196 in exon 1, changing His to Gln at aa64. Both alleles are missense mutations and therefore might only reduce the function of the protein mildly.

op325, the new allele of *ced-10*, was sequenced by Kelvin Wong, a postdoc in the lab. *ced-10(op325)* mutant worms have an A to C transversion at position 14 in exon 1, changing Lys to Thr at aa5. This missense mutation does not lie within a specific domain of CED-10 and therefore might not result in a strong loss of function of the protein.

3.3. Discussion

At least seven genes have been identified that promote engulfment of apoptotic cell corpses in *C. elegans*. Genetic studies grouped the known engulfment genes into two partially redundant pathways, which converge at the level of *ced-10*. Interestingly, none of the known engulfment genes is absolutely essential for cell corpses engulfment as many apoptotic cells are still properly removed in mutant animals. However, beside profound genetic epistasis analysis and few additional subcellular localisation studies, most of the engulfment genes have been poorly characterised. Importantly, the fate of unengulfed cell corpses has never been examined thoroughly. In this study, I performed a time course analysis of the engulfment defect caused by mutations in known engulfment genes by scoring the number of persistent cell corpses during development of mutant worms. My results clearly demonstrate that persistent apoptotic cell corpses do not last forever as they are slowly removed over time. Interestingly, I found that although the number of persistent cell corpses differs in the distinct engulfment mutants, their decay curves are quite similar. The half-life of persistent cell corpses is in the range of 3-6 days. Furthermore, comparison of single and double engulfment mutants indicated that persistent cell corpses in single engulfment mutants are not removed through the other, parallel engulfment pathway. But how these corpses are removed remains unclear. While most of the engulfment mutants displayed similar decay curves *ced-7* mutants showed a dramatically distinct behaviour: in *ced-7* mutant animals, unengulfed cell corpses hardly persist during larval development, resulting in a much shorter half-life. Thus, even in the absence of *ced-7*, a mechanism exists that can promote relatively efficient apoptotic cell corpse removal during larval development. It is tempting to postulate the existence of an alternative engulfment pathway, which might act as a backup mechanism to prevent the accumulation of persistent cell corpses that might have deleterious consequences. But whether this ‘salvage pathway’ is only specific for *ced-7* and whether it acts mainly during larval development remains to be determined. Certainly, further

experiments need to be done to uncover the mysterious nature of this putative 'salvage pathway'.

A good approach to identify novel components of a signalling pathway is to perform a genetic screen. As I focussed my interest on *ced-7*, I decided to do an ENU mutagenesis in a *ced-7* mutant background and screened for worms with an enhanced engulfment defect during larval development. The screen was successful in the sense that I isolated eight mutants, which significantly enhanced the number of unengulfed cell corpses in *ced-7* mutant larvae. Unfortunately, all mutants failed to complement alleles of known engulfment genes that had been found in previous screens. Nevertheless, based on the fact that this screen was not saturated, this result does not exclude the existence of further engulfment genes that promote the removal of persistent cell corpses in *ced-7* mutant larvae. Rather, our findings confirmed the feasibility of the screen.

How can one explain the high frequency of mutations in the gene *ced-1*? ENU produces a broad mutagenic spectrum and causes an average knockout frequency of about 1 in 4000 genomes [17]. But how many times a single gene is hit by a specific mutagen also depends on its size. By comparing the size of *ced-1* to other genes in the *C. elegans* genome, it becomes evident that *ced-1* is rather a large gene: genomic *ced-1* comprises ~16,5kb and consists of 11 exons. The probability of being hit by ENU is therefore higher for *ced-1* than for other genes and might explain the high mutation rate of *ced-1*.

The two alleles of *ced-2* isolated in the screen most likely reduce the function of the protein only mildly. Both alleles are missense mutations that cause the replacement of His, an imidazole ring containing amino acid by Gln, a neutral amino acid. *op321* changes His to Gln at aa98 at the penultimate amino acid of the SH2 domain; most probably this substitution does not impair the adaptor function of CED-2. *op327* replaces His by Gln at aa64 in the middle of the SH2 domain and might therefore have a greater impact on the adaptor function of CED-2. However, it is unlikely that *op327* impairs the binding of CED-2 to CED-5 since it has been reported that the C-terminal

region of CED-5, which contains a SH3-binding proline-rich domain, interacts with CED-2 [16].

op325 encodes a new allele of *ced-10*, resulting in a Lys to Thr change at aa5. Although the mutation affects an early amino acid in the first exon, it does not alter any conserved domain of CED-10. Replacement of Lys, a basic amino acid by Thr, an aromatic amino acid might cause a stereotypic alteration of the protein that might reduce its signalling capacity. Surely, *op325* is not a null allele, since worms homozygous for *ced-10(t1875)*, a probable null mutation, are not viable (Kinchen et al., *unpublished*).

Considering the fact that I only found previously identified engulfment genes in the performed *ced-7* enhancer screen, the question raises whether any other genes are required for engulfment in *C. elegans* at all? The answer is certainly yes. Several lines of evidence suggest the existence of further, but as yet unidentified engulfment genes: None of the known engulfment genes is absolutely essential for the engulfment process, as many dying cells are still properly removed in mutant animals. Even in a strong double mutant between genes of the two distinct engulfment pathways, a few cell corpses are always properly engulfed. These data indicate that the engulfment defect, caused by the known engulfment mutants, can be bypassed by as yet unknown mechanisms. The assumption that other engulfment genes exist is not only supported by genetic but also by molecular data: The proteins identified so far constitute only parts of the signalling pathway: receptors and intracellular signalling complexes. The ‘eat-me’ signal that labels the dying cell for engulfment as well as downstream target molecules leading to the remodelling of the actin cytoskeleton have not been identified yet. Recently, the PS receptor PSR-1 has been proposed to play a role in the engulfment of apoptotic cell corpses in *C. elegans*, but the data remain conflicting. Although Wang et al. (2003) reported in their paper that PSR-1 mediates cell corpse engulfment through CED-5 and CED-12 [18], I was not able to reproduce the engulfment phenotype of *psr-1* mutant worms (Chapter 4) and recent data from Cui and colleagues (2004) implicate that mammalian PSR localises to nuclei through multiple nuclear localisation signals, demonstrating at least one

additional function of mammalian PSR beside its proposed role in phagocytosis at the cell surface [19]. Another protein that localises to the plasma membrane and that has been proposed to encode a receptor for the 'eat-me' signal on the dying cell is CED-1 [20]. However, the appropriate ligand of CED-1 has not been identified yet. On the other hand, downstream targets of *ced-10* that trigger actin polymerisation, a process required for efficient phagocytosis, are still unknown. The published targets of *ced-10* (*gex-2, gex–3* and genes of the Arp2/3 complex) are required for cell migration during morphogenesis but have not been shown to play a role in the engulfment of apoptotic cells [21,22]. Together, these data strongly suggest that extra genes exist, promoting the engulfment of apoptotic cells in *C. elegans*.

Assuming that additional engulfment genes exist, why didn't I find any of them in the *ced-7* enhancer screen? First of all, I have to point out that the screen was not saturated. I only screened 10'000 haploid genomes due to the laborious screening method (Nomarski screen). It is most likely that I would have isolated more engulfment mutants in a larger screen.

Redundancy might be another explanation for my inability to identify novel engulfment genes in the *ced-7* enhancer screen. In case of redundancy, abrogating the function of a single gene might not be sufficient to cause any phenotype since the unaffected redundant gene can substitute for the loss of the other gene. Usually, the method to avert redundancy is to screen for new mutations in a sensitive mutant background. Theoretically, *ced-7* appears to be an ideal mutant background since *ced-7* encodes a transmembrane protein and has been placed upstream in the engulfment cascade. In addition, double mutant analysis between *ced-7* and other engulfment genes has shown that the mutant larval phenotype of *ced-7* can easily be enhanced through mutations in other engulfment genes. However, this putative advantage turned out to be a drawback as I only isolated new alleles of previously identified engulfment genes.

The third reason for the failure of the screen might be lethality. Obviously, lethal genes could not have been identified in the way the *ced-7*

enhancer screen has been performed. Indeed, one can easily imagine that some engulfment genes, which are involved in the reorganisation of the cytoskeleton might cause embryonic lethality when knocked out during morphogenesis. A good example of an engulfment gene that results in maternal effect embryonic lethality is *ced-10(t1875)*. The *t1875* allele has been isolated in a genetic screen for mutations that result in embryonic lethality on chromosome IV, resulting in a lesion that alters the initial ATG (translational start site) (Kinchen et al., *unpublished*). As this allele is most likely a true null allele of *ced-10*, one can assume that loss-of-function mutations in other engulfment genes might cause embryonic lethality too.

Taken together, multiple circumstances might have led to the pitfall of the screen. Since screens done by Nomarski optics are very time consuming, it might be worthwhile to develop new screening strategies that allow high throughput screening using a standard dissecting microscope. Nonetheless, several lines of evidence suggest the existence of a 'salvage pathway' through which persistent cell corpses are finally removed – at least in *ced-7* mutant animals. Revealing the nature of this pathway might provide new insights into engulfment mechanisms not only in worms but also in humans.

3.4. Materials and Methods

Mutations and Strains

Methods for culturing *C. elegans* strains have been described previously [23]. Unless otherwise stated, worms were grown at 20°C. All mutants used in this study were derived from the wild-type variety Bristol strain N2. The following mutations are described in Riddle et al. (1997) [24], except the *ced-12* alleles *bz187* and *oz167*, which have been described elsewhere [5]. LGI: *ced-1 (e1735)*, *ced-12(bz187)*, *ced-12(oz167)*. LGIII: *ced-6(n1813)*, *ced-6(n2095)*, *ced-7(n1996)*, *ced-7(n1892)*, *unc-16(e109)*. LGIV: *ced-2(e1752)*, *ced-5 (n1812)*, *ced-10(n1993)*, *ced-10(n3246)*. Double mutant strains used in this study were obtained from the CGC (*ced-1(e1735); ced-10(n1993)*), from R. Horvitz (*ced-1(e1735); ced-5(n1812)*, *ced-5(n1812); ced-7(n1892)*, *ced-5 (n1812); ced-10(n1993)*, and *ced-6(n1813); ced-2(e1752)*) or built in the Hengartner laboratory (*ced-1(e1735); ced-7(n1892)* and *ced-5(n1812); ced-12 (oz167)*) using standard genetic approaches.

Cell Corpse Assay

Persistent cell corpses were scored in whole embryos at early developmental stages and in the head region from the early three fold stage onward. Mixed staged worms were mounted on 4% agarose pads in a drop of M9 salt solution containing 30mM NaN_3 and analysed by Nomarski optics using a Leica DMRA microscope. Persistent cell corpses appear as highly refractile disks and can readily be identified under high magnification. Statistical analysis was performed using StatView version 4.5 software (Abacus Concepts, Incorporated, Berkely, California).

ENU Mutagenesis of *ced-7* Mutant Worms

N-ethyl-*N*-nitrosourea (ENU) mutagenesis was performed as previously described [25]. Briefly, *ced-7(n1996)* mutant hermaphrodites were washed off with M9 buffer and transferred into a conical tube. All but 4ml M9 buffer was removed and ENU (Sigma), dissolved in 99.8% EtOH to a final concentration of 100mM, was added to a final concentration of 1mM. Worms were incubated

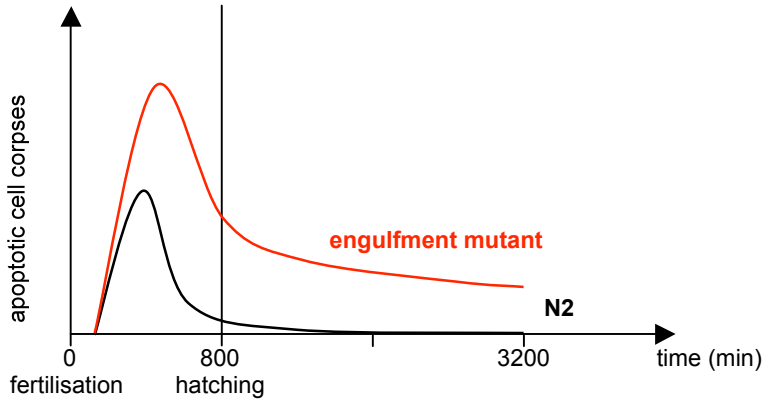
in the ENU solution for 4 hours with gentle agitation and then washed 2x with M9 buffer and placed on a seeded plate at 15°C overnight. The next day, mutagenised adult worms were picked to multiple large seeded plates and allowed to lay eggs for approximately 5 hours. Adult hermaphrodites were then removed from the plate. Once the F2 progeny reached adulthood and produced offspring themselves, they were washed off the plate to avoid overcrowding and to synchronise the F3 generation. L3 larvae were mounted on 4% agarose pads in a drop of M9 buffer containing 30mM NaN_3 and observed by Nomarski microscopy for an enhanced engulfment defect. Approximately 10'000 haploid genomes were screened for recessive, maternally-rescued mutations; this number corresponds to ~46'000 haploid genomes for recessive, zygotic mutations. Candidate mutants were recovered by picking them from the agarose pad and transferring them to a fresh seeded plate (1 worm / plate).

Genetic Mapping and Complementation Tests

New mutations that bred true were backcrossed 3x to the parental *ced-7 (n1996)* strain. To analyse whether the mutant phenotype persists in a *ced–7(+)* background, *ced-7* was removed using the closely linked visible marker *unc-16(e109)*. Mutations have been mapped to chromosomes by standard three-factor mapping. Complementation tests were performed with different alleles of known engulfment genes.

3.5. Figures and Tables

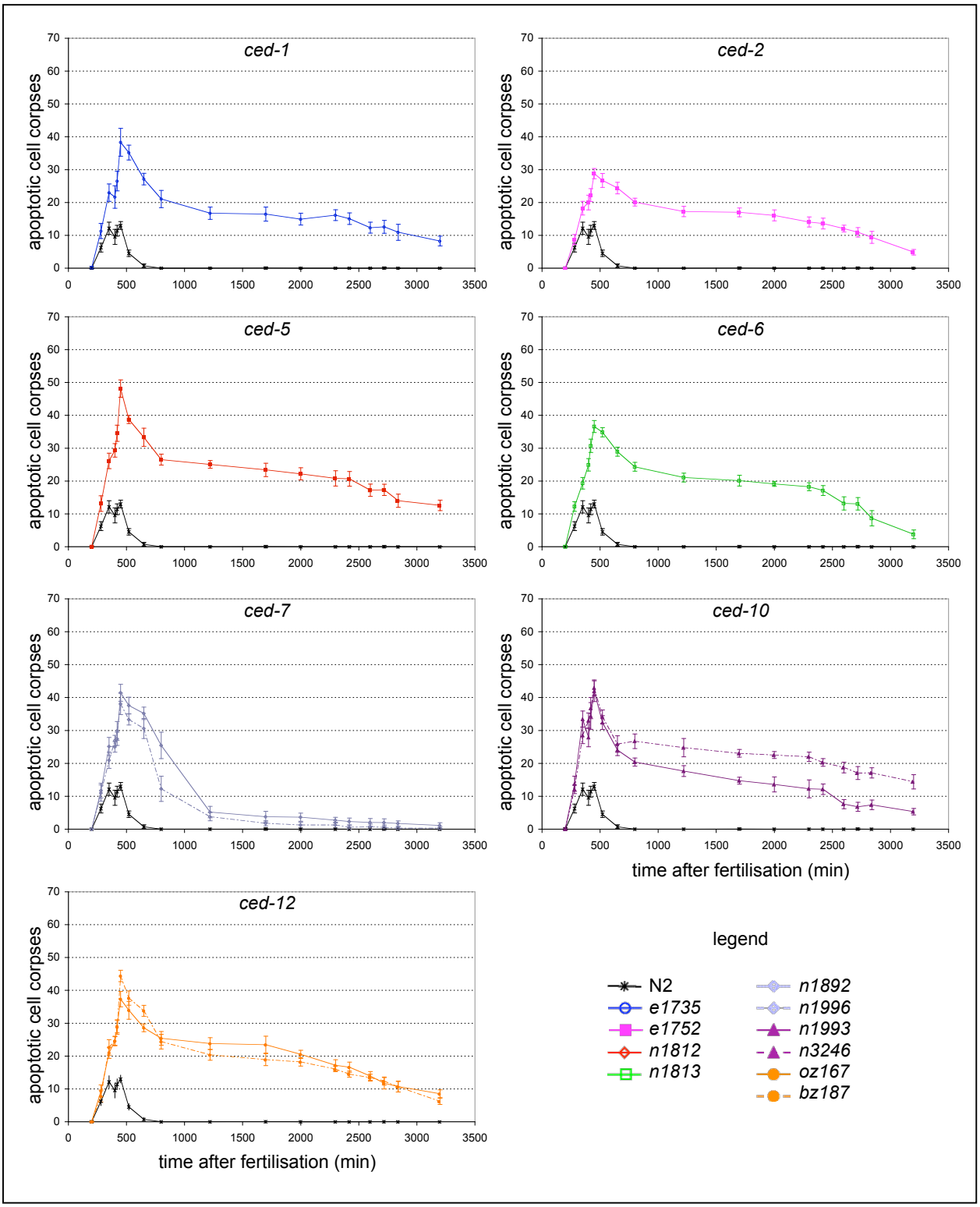
Figure 1: Schematic Outline of Apoptotic Cell Corpse Patterns in Wildtype and Engulfment Mutants



Developmental Stage:	**Embryogenesis**		**Larval Development**	
Time after fertilisation:		(min)		(min)
	ball of cells	(200)	early L1	(800)
	tramtrack	(280)	mid L1	(1220)
	U-view	(350)	late L1	(1700)
	comma	(400)	early L2	(2000)
	1.5 fold	(420)	late L2	(2300)
	2 fold	(450)	early L3	(2420)

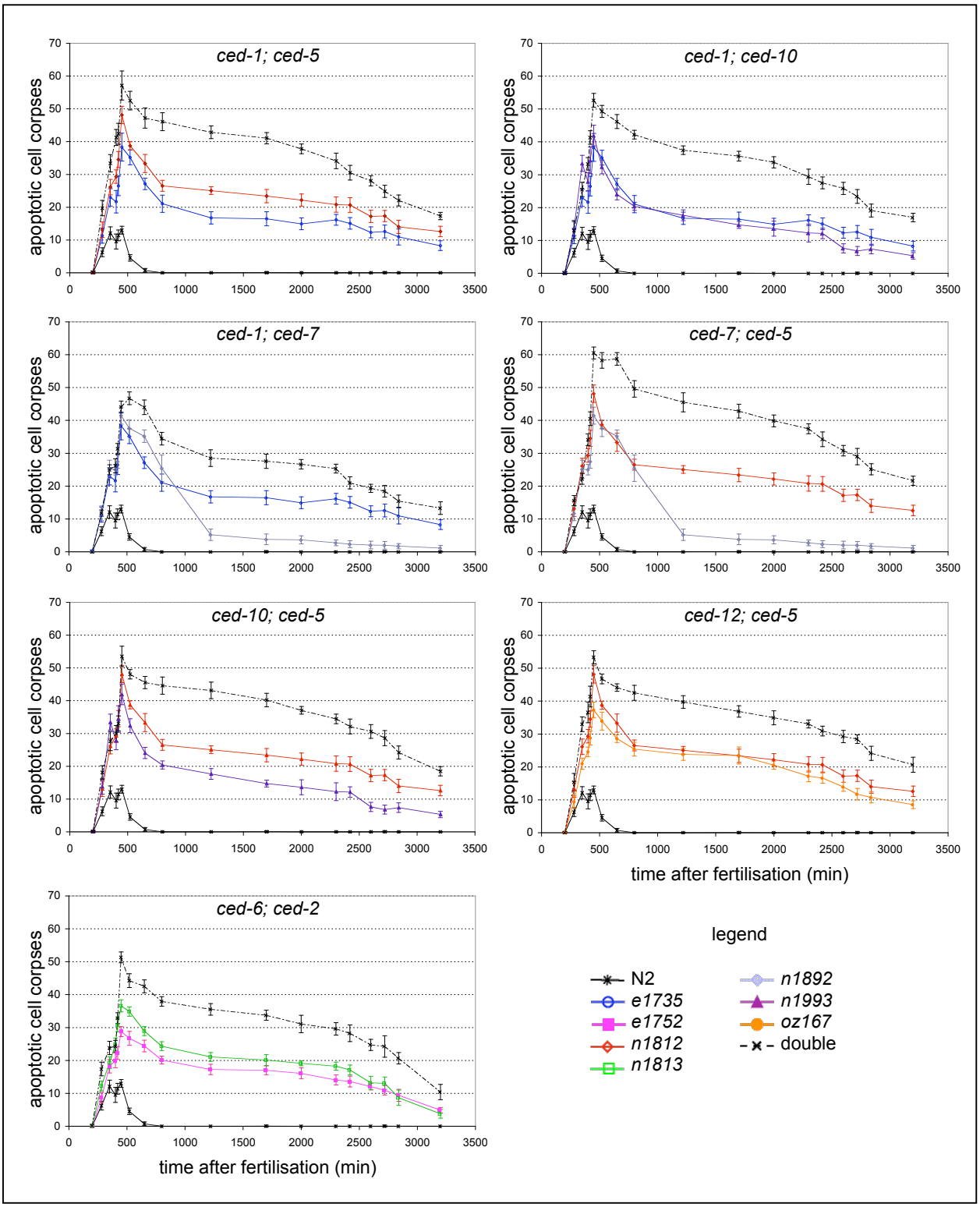
To precisely follow the persistence of apoptotic cell corpses over time, the embryonic and larval stages defined by Sulston et al. (1983) were divided into 18 subclasses, which can be distinguished by Nomarski microscopy.

Figure 2: Apoptotic Cell Corpses Persist in Engulfment Mutants



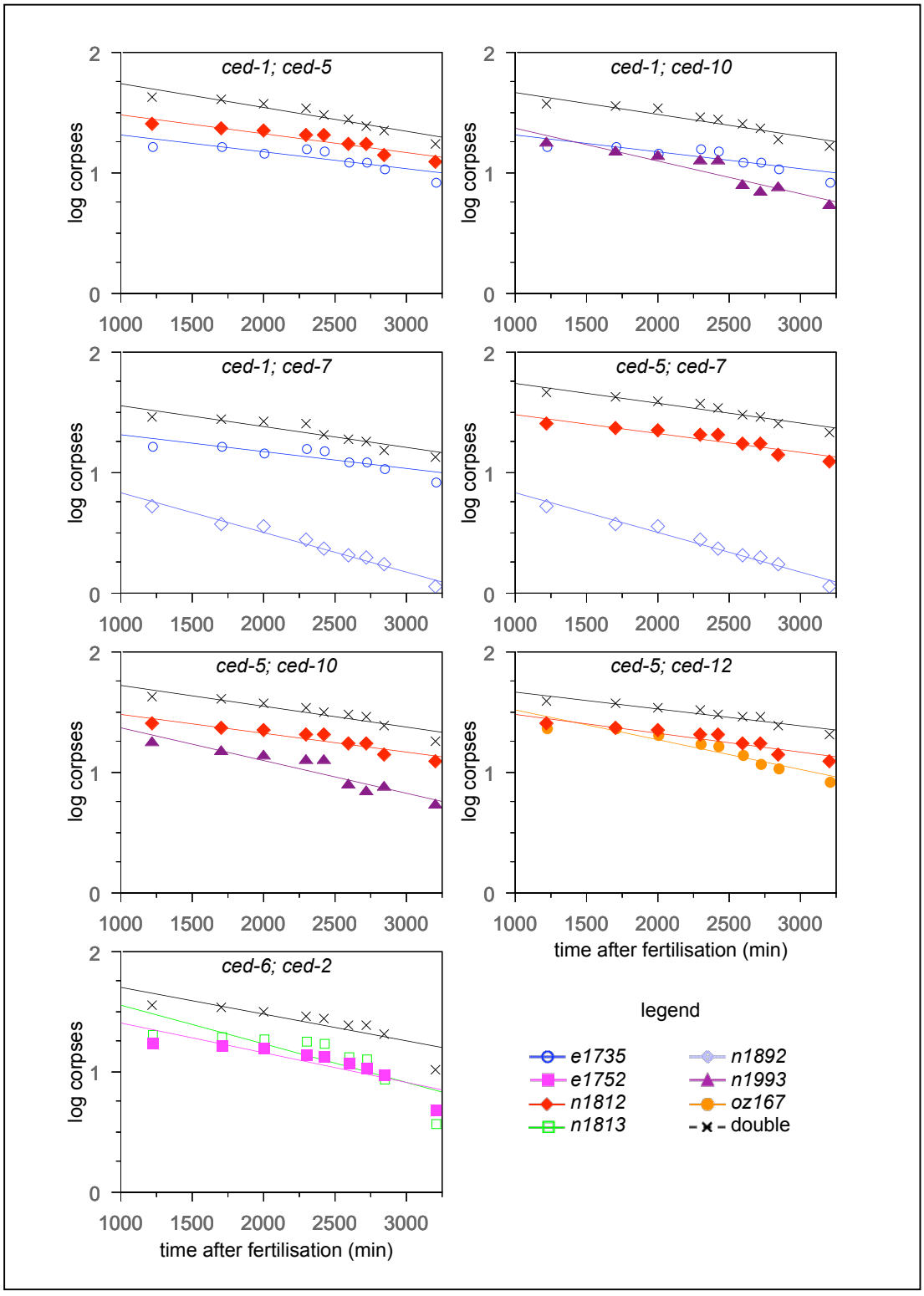
Persistent cell corpses were scored in whole embryos at early developmental stages and in the head region from the early three fold stage onward. At least one allele of each engulfment gene was scored. All data are averages ± standard deviation (SD), n=15.

Figure 3: Double Mutants Between the Two Engulfment Groups Synergistically Increase the Engulfment Defect but Do Not Abolish the Loss of Persistent Cell Corpses



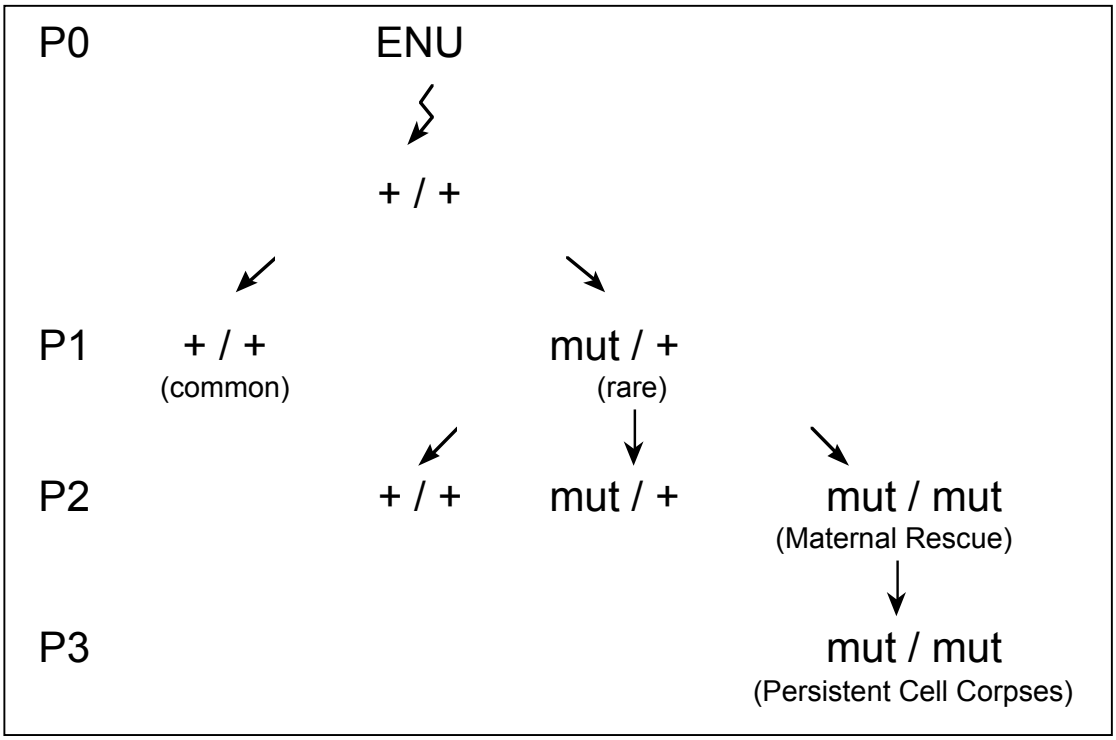
Persistent cell corpses were scored in whole embryos at early developmental stages and from the early three fold onward only in the head region. All data are averages ± standard deviation (SD), n=15.

Figure 4: Single And Double Engulfment Mutants Show Similar Kinetics of Persistent Cell Corpse Elimination



Regression plot analysis was performed on persistent cell corpse numbers from the mid L1 larval stage onward, using StatView version 4.5 software.

Figure 5: Schematic Outline of the *ced-7(n1996)* Enhancer Screen



ENU mutagenesis was performed as described in Materials and Methods. L3 larvae of the F3 generation were screened for persistent cell corpses in the head region.

Table 1: Regression Plot Analysis of Persistent Cell Corpses

genotype	cell corpses in early L1s	y intercept	halflife (1:x) days	R^2
N2	0			
ced-1(e1735)	21.1 ± 2.6	1.444	125	0.809
ced-2(e1752)	20.1 ± 1.2	1.542	83	0.731
ced-5(n1812)	26.5 ± 1.7	1.574	125	0.847
ced-6(n1813)	24.3 ± 1.3	1.714	67	0.647
ced-7(n1996)	12.3 ± 3.8	1.441	26	0.949
ced-7(1892)	25.6 ± 4.0	1.505	36	0.879
ced-10(n1993)	20.4 ± 1.2	1.565	67	0.891
ced-10(n3246)	26.7 ± 2.2	1.537	167	0.894
ced-12(bz187)	24.4 ± 2.2	1.497	63	0.826
ced-12(oz167)	25.4 ± 2.1	1.647	83	0.87
ced-1(e1735); ced-5(n1812)	46.0 ± 2.7	1.864	98	0.868
ced-1(e1735); ced-10(n1993)	42.2 ± 1.3	1.798	104	0.889
ced-1(e1735); ced-7(n1892)	34.5 ± 1.9	1.698	103	0.881
ced-6(n1813); ced-2(e1752)	37.9 ± 1.4	1.805	94	0.676
ced-7(n1892); ced-5(n1812)	49.6 ± 2.5	1.856	114	0.895
ced-10(n1993); ced-5(n1812)	46.6 ± 2.6	1.824	115	0.837
ced-12(oz167); ced-5(n1812)	42.5 ± 2.3	1.760	138	0.886

Numbers of persistent cell corpses in early L1s are averages ± standard deviation (SD). Regression plot analysis was performed using Statview version 4.5 software.

Table 2. New Mutations Found in the *ced-7(n1996)* Enhancer Screen

Mutation	Gene	Chromosome	No. of cell corpses*
control			0.6 ± 0.6
op322	*ced-1*	I	15.8 ± 1.6
op323	*ced-1*	I	11.6 ± 1.8
op324	*ced-1*	I	15.4 ± 1.2
op365	*ced-1*	I	8.5 ± 1.2
op379	*ced-1*	I	20.0 ± 2.0
op321	*ced-2*	IV	6.9 ± 0.9
op327	*ced-2*	IV	17.5 ± 2.3
op325	*ced-10*	IV	22.8 ± 1.7

Persistent cell corpses were counted in the head region of L3 larvae. Data shown are averages ± standard deviation (SD), n=15. * All strains were also homozygous for *ced-7(n1996)*. *ced-7(n1996)* control worms do not show any corpses at the L3 stage.

3.6. References

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Chapter 4

C. elegans P-type ATPase TAT-1 Is Required for Phosphatidylserine Exposure and Efficient Engulfment of Apoptotic Cells

4.1. Introduction

In most eukaryotic cells, the phospholipids of the plasma membrane are nonrandomly distributed between the two leaflets of the bilayer [1,2]. The outer leaflet of healthy cells primarily contains the choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (Sph), while the inner leaflet mainly contains the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS). Loss of phospholipid asymmetry and the consequent exposure of PS on the cell surface appears to be a universal feature of apoptotic cells and serves as a signal for the assembly of the protease complexes of blood coagulation [3] as well as the recognition of apoptotic cells by phagocytes [4,5]. PS is normally restricted to the cytosolic leaflet of the plasma membrane by an enzymatic ATP-dependent activity, called the aminophospholipid translocase. This activity has been purified from human erythrocytes [6], bovine chromaffin granules [7], and synaptic vesicles [8] and has been suggested to be equivalent to ATPase II, originally isolated from bovine chromaffin granules [9]. Cloning of the aminophospholipid translocase gene confirmed that it encodes an ATPase II, which belongs to the subfamily of P-type ATPases [10]. Sequence analysis revealed striking similarity to yeast DRS2, and up to now most studies of ATPase II have been done in the yeast *Saccharomyces cerevisiae*.

Until recently, data concerning the function of *drs2* has been quite contradictory: some researchers have reported a decrease of spin-labelled derivatives of PS in *drs2*Δ strains [10], whereas others have not found any role for Drs2p in aminophospholipid translocation [11]. However, new findings shed some light on the cause of the disparity in data on lipid translocation across the *drs2* mutant plasma membrane: Drs2p mainly localises to late Golgi membranes, indicating that the primary site of Drs2p function is in the trans-Golgi network (TGN) rather than the plasma membrane [12]. *drs2*Δ mutant exhibit significant defects in protein transport from the TGN, similar to clathrin mutants, which could cause the misslocalisation or inactivation of proteins at the plasma membrane [12-14]. Notably, the yeast genome contains four genes

closely related to DRS2, namely NEO1, DNF1, DNF2, and DNF3 [14,15]. Combined mutant analysis revealed that Drs2p, Dnf1p, Dnf2p and Dnf3p constitute an essential protein family with overlapping functions in membrane trafficking between the Golgi and the endosomal/vacuolar system [14]. Dnf1p and Dnf2p are both restricted to the plasma membrane and are essential for inward translocation of NBD-PE, -PS and -PC across the plasma membrane [16]. Loss of Dnf1p or Dnf2p results in the exposure of PE on the outer leaflet of the plasma membrane, but since *dnf1* and *dnf2* mutants also perturb protein localisation, the possibility that the translocation defect is a secondary effect of the mutations can not be ruled out [14,16]. A recent publication by Natarajan and colleagues (2004) [17] demonstrates convincingly that Drs2p specifically translocates fluorescent-labelled PS from the luminal to the cytoplasmic leaflet of the TNG. Interestingly, *cho-1* yeast strains that are defective in PS synthesis do not phenocopy *drs2*, but instead transport proteins normally via the secretory pathway. This observation suggests that translocation of PS is not required for the function of Drs2p in protein transport from the TGN and that Drs2p, although PS is a preferred substrate in vitro, displays different substrate specificity in vivo.

Efficient elimination of apoptotic cells is crucial for the integrity of multicellular organisms. Unengulfed apoptotic cell corpses can undergo secondary necrosis causing the release of potentially cytotoxic and antigenic intracellular contents into the surrounding tissue. Accordingly, defects in the clearance of apoptotic cells have been suggested to contribute to the development of chronic inflammation and autoimmunity disorders [18]. Much of today's knowledge regarding engulfment of apoptotic cells comes from studies in the nematode *C. elegans*. At least seven genes – *ced-1*, *-2*, *-5*, *-6*, *-7*, *-10*, and -12 – have been identified that play a crucial role in the engulfment of apoptotic cell corpses in *C. elegans* [19-21]. Importantly, all engulfment genes are evolutionarily conserved in humans, underlining the value of *C. elegans* as a model organism.

Recently, Wang and colleagues reported the cloning and characterisation of *psr-1*, the worm homologue of PSR [22]. Similarly to its

mammalian homologue, PSR-1 preferentially binds PS and mediates the engulfment of apoptotic cell corpses. Interestingly, *psr-1* seems to act in the same signalling cascade as *ced-2*, *-5*, *-10*, and *-12*. Notably, although the published data indicates a function for *psr-1* in the removal of apoptotic cell corpses, the authors missed to demonstrate that apoptotic cells in *C. elegans* actually do expose PS. Since recent data report nuclear localisation of PSR through multiple nuclear localisation sites [23] and my own results contradict the published engulfment defect of *psr-1* mutants worms (Züllig and Hengartner, unpublished; Supplementary Figure 1), the issue of PS exposure on apoptotic cells in *C. elegans* acquires great importance.

In order to better understand the mechanisms of PS exposure and its role in the clearance of apoptotic cells in vivo, I examined PS exposure in *C. elegans*. PS exposure appears to be an evolutionarily conserved feature of apoptosis, and has been described in mammalian, avian, amphibian, fish, and even invertebrate species such as *Drosophila melanogaster* [24-27]. Remarkably, however, it has never been demonstrated yet, whether apoptotic cells in *C. elegans* expose PS as well. To address this question I took advantage of a well-established mammalian cell culture assay, namely the labelling of apoptotic cell corpses by Annexin V (AnxV) [28]. AnxV is a Ca^{2+}-dependent aminophospholipid-binding protein with high affinity for PS that has been proven to be a sensitive marker for PS exposure on the cell membrane of dying cells [29,30].

4.2. Results

To determine whether PS is exposed on apoptotic cells in worms as well, I built an inducible GFP::AnxV reporter construct that is expressed by all cells in *C. elegans* and is secreted into the extracellular medium (Supplementary Figure 2). Using this in vivo approach, I found that dying cells in *C. elegans* do expose PS, as shown by the selective highlighting of cell corpses by GFP in embryos and the germ line of adult *opIs117* hermaphrodites (Figure 1). However, not every single cell corpse that is detectable by Nomarski optics, is marked by GFP::AnxV. This discrepancy can be explained by two possible mechanisms: (I) The GFP::AnxV fusion protein does not access every cell corpse to the same extent, possibly due to restricted protein secretion. (II) Apoptotic cell corpses with the characteristic appearance of a refractile disk are already in a late engulfment stage where the plasma membrane becomes disassembled and proteins become degraded inside the engulfing cell, resulting in a loss of GFP::AnxV staining.

To demonstrate that the labelling of apoptotic cell corpses by GFP::AnxV is specific, I generated a GFP control construct that is expressed by the same promoter and carries the same secretion signal as the GFP::AnxV reporter construct. As expected, worms carrying the control transgene *opIs172* express GFP in a similar expression pattern as transgenic *opIs117* animals, but apoptotic cell corpses are never labelled with GFP (Table 1). Taken together, these findings strongly suggest for the first time that, like mammalian cells, *C. elegans* cells also expose PS at the onset of apoptosis.

In order to determine at which stage of engulfment GFP::AnxV labels apoptotic cells, I co-stained transgenic *gla-1(op324)* worms, which have an increased number of physiological cell death in the adult hermaphrodite germ line (Milstein and Hengartner, *unpublished*), with the Hoechst 33342 dye to visualise the DNA condensation of apoptotic cells and with the SYTO41 dye, which stains engulfed cells (Kinchen et al., *unpublished*). Engulfment begins at an early time point in the apoptotic program, concomitant with induction of

DNA degradation [31]. I found that GFP::AnxV preferentially stains cells in the early stages of apoptosis, when the DNA becomes condensed, but the cell appears only mildly refractile by DIC and engulfment is not completed yet (Figure 2). Late, engulfed (SYTO41+) apoptotic cells invariably failed to stain with GFP::AnxV. Thus, PS exposure is an early event in apoptosis.

Is PS exposure truly dependent on apoptosis? In order to answer this question, I crossed the GFP::AnxV transgene into different apoptosis mutant backgrounds and examined GFP::AnxV staining in the adult hermaphrodite germ line (Table 1). Notably, mutants that are defective in the cell death genes *ced-4* or *ced-3*, which result in almost complete abrogation of apoptotic cell death, do not show any specific pattern of GFP::AnxV staining, suggesting that cell death is required for PS exposure in *C. elegans*. Conversely, increase of apoptotic cell death caused by the loss of the Bcl-2 homologue *ced-9* or a loss-of-function mutation in the *gla-1* gene results in an increased number of apoptotic germ line corpses stained with GFP::AnxV. Thus, PS exposure and labelling of apoptotic cell corpses by GFP::AnxV depends on apoptotic cell death.

Notably, the asymmetric distribution of phospholipids is not only lost during apoptotic but also during necrotic cell death when the plasma membrane becomes disrupted and the cell lyses [32]. Consequently, if GFP::AnxV binds specifically to negatively charged aminophospholipids such as PS and PE, one would expect it to label necrotic cell corpses too. To test this hypothesis, I analysed GFP::AnxV labelling of necrotic cell corpses in a *mec-4(u231)* mutant background. The dominant *mec-4(u231)* mutation hyperactivates the Na^{2+} channel in the six *C. elegans* touch-receptor neurons, causing vacuolar swelling and lysis of the specific neurons. Consistent with a high substrate specificity of AnxV, I found that GFP::AnxV also labels necrotic cells in the tail of *C. elegans* larvae. Importantly, unlike apoptotic death, labelling of necrotic cell deaths by GFP::AnxV was not suppressed by mutations in the caspase *ced-3* (Table 2, Figure 3). These results support the hypothesis that GFP::AnxV specifically binds to negatively charged

aminophospholipids (most likely PS) that become accessible only after apoptotic or necrotic cell death.

In mammals, the CED-7 homologue ABCA1 *(ATP-b*inding-*c*assette transporter A1) has been implicated in the engulfment of apoptotic cells [33]. Macrophages derived from ABCA1$^{-/-}$ mice show reduced clearance of apoptotic cells and forced expression of ABCA1 confers increased engulfment ability to non-phagocytic cells [34]. Interestingly, in vitro gain-of-function experiments also showed that ABCA1 promotes the release of cellular cholesterol and the redistribution of PS in the transmembrane bilayer of ABCA1 transfected cells. Therefore, I tested whether *ced-7* or any of the other known *C. elegans* engulfment genes is necessary for PS exposure. To this end, I crossed the GFP::AnxV transgene into all known engulfment mutants and assayed GFP::AnxV labelling of apoptotic germ cell corpses (Figure 4). To our surprise, *ced-7*, as well as all the other engulfment genes tested, appear not to be required for PS exposure in *C. elegans.* Importantly, in the engulfment defective backgrounds, the number of corpses stained by GFP::AnxV always exceeded the number of corpses counted by DIC optics. Thus, in these mutants, GFP::AnxV labelled not only early, but also mature (unengulfed) cell corpses.

What genes are required for PS exposure in *C. elegans*? Previous studies reported the existence of a Ca^{2+}-dependent, bidirectional phospholipid transfer activity, termed the scramblase, that mediates the translocation of aminophospholipids across the bilayer of activated, injured or apoptotic human erythrocytes and blood platelets [35,36]. The erythrocyte scramblase PLSCR1 (*p*hospho*l*ipid *scr*amblase 1) has been cloned [37] and since then several additional isoforms have been identified [38]. However, blood cells derived from PLSCR1$^{-/-}$ mice mobilise PS to the cell surface upon stimulation as efficiently as wild-type cells, querying the proposed role of PLSCR1 as a scramblase [39]. Additionally, mice with targeted deletion of PLSCR3 aberrantly accumulate adipocyte fat stores, a phenomenon that is associated with the onset of dyslipidemia and insulin resistance [40]. However, the authors did not mention any defects in plasma membrane phospholipids scrambling of

apoptotic cells derived from PLSCR3$^{-/-}$ mice. To test whether scramblases might be involved in lipid redistribution in *C. elegans*, I knocked down the expression of 10 annotated scramblases by RNAi [41-44]. PS exposure was analysed using the GFP::AnxV reporter construct *opIs117* (Figure 5). I did not observe any reduction of GFP::AnxV labelling around apoptotic cell corpses, either in wild-type or engulfment defective worms. This experiment suggests that none of the putative scramblases is essential for PS exposure in *C. elegans*. However, these negative results should be interpreted very cautiously. First, the efficiency of RNAi is highly variable; it is thus possible that our RNAi treated worms still expressed significant levels of scramblase mRNA. Second, I only did single gene RNAi; I therefore cannot exclude the possibility that two or more scramblase genes act redundantly to promote PS exposure.

Other genes with a putative function in the translocation of aminophospholipids across the membrane bilayer are the aminophospholipid translocases. Aminophospholipid translocases belong to the subfamily of P-type ATPases and are known to mediate the inwardly directed, ATP-dependent transport of aminophospholipids (flippases) [10]. The *C. elegans* genome encodes six putative P-type aminophospholipid transporters [45], which we have named *tat-1*, *-2*, *-3*, *-4*, *-5* and *-6* for *t*ransbilayer *a*mphipath *t*ransporter 1-6 (Table 3). To test their function in the translocation of aminophospholipids across the plasma membrane of apoptotic cells, I ablated the function of *tat-1*, *-2*, *-3*, *-4*, and *-5* by RNAi and scored the number of GFP::AnxV labelled cell corpses in the adult hermaphrodite germ line. Surprisingly, ablating the function of *tat-1* by RNAi caused a dramatic reduction in the number of GFP::AnxV labelled cell corpses, indicating a putative role for *tat-1* in PS exposure in *C. elegans* (Figure 6). Importantly, the same effect was observed by feeding a different *tat-1* RNAi expression vector (pSZ3) that targets another part of the *tat-1* mRNA for degradation, confirming the specific impact of *tat-1(RNAi)* on PS exposure (Figure 6, *tat-1(pSZ3)*).

The requirement of *tat-1* in PS exposure was first observed in a *ced-5* mutant background. To determine whether the *ced-5*(lf) background was

specifically required for this effect, the experiment was repeated using mutants for the other engulfment genes. Notably, RNAi on *tat-1* abolished GFP::AnxV staining around cell corpses in all the different engulfment mutants (Figure 7). These results demonstrate that the phenotype caused by *tat-1* RNAi treatment does not depend on a particular mutant engulfment background.

Assuming that PS represents the 'eat-me' signal for phagocytosis of apoptotic cells in *C. elegans* as well, one might expect an engulfment defect in *tat-1(RNAi)* worms. To test this hypothesis, I scored unengulfed cell corpses in *tat-1(RNAi)* worms during embryonic and larval development as well as in the adult hermaphrodite germ line (Figures 8 and 9). Loss of *tat-1* function by RNAi did not affect the clearance of apoptotic cell corpses during embryonic or larval development since the number of unengulfed cell corpses in *tat-1*(*RNAi)* worms is not significantly enhanced. However, *tat-1(RNAi)* resulted in a weak engulfment defect in the germ line of adult *opIs117* hermaphrodites. At the same time, the number of GFP::AnxV labelled germ line cell corpses was reduced. There could be multiple reasons for the different effect of *tat-1* loss-of-function in the adult hermaphrodite germ line compared to embryonic and larval development. First, since the efficiency of RNAi differs in various tissues, it could be that the *C. elegans* germ line is simply more sensitive to RNAi. Another explanation might be redundancy: if another gene exerts the same function as *tat-1* during development, abrogating *tat-1* function would not cause an engulfment phenotype at this specific developmental stage. A third possibility is that *tat-1* is not expressed during embryonic and larval development, indicating a function for *tat-1* in PS exposure exclusively on apoptotic cell corpses in the germ line. The last hypothesis seems to be questionable, as *in situ* hybridisation data from the Kohara lab indicates that *tat-1* is expressed not only in the adult hermaphrodite germ line, but also during embryonic development (NEXTDB, Ver.3.0 beta Jul 07, 2001).

In order to check whether the phenotypic differences of *tat-1(RNAi)* are the result of distinct RNAi efficiencies, I repeated the *tat-1(RNAi)* experiments

in *rrf-3* (*R*NA-dependent *R*NA polymerase *f*amily 3) mutant worms. *rrf-3* encodes a putative RNA-directed RNA polymerase and *rrf-3* mutants are hypersensitive to RNAi [46]. Interestingly, in the *rrf-3* mutant background *tat-1 (RNAi)* results in an enhanced number of apoptotic cell corpses during embryonic development (Figure 10). Likewise, *tat-1* loss-of-function in *rrf-3* mutant worms causes an increase in the number of unengulfed cell corpses in the hermaphrodite germ line (Figure 11). These findings suggest that *tat-1* plays a role in the removal of apoptotic cell corpses, most likely due to its function on PS exposure.

It is worth noting that the phenotype of enhanced apoptotic cell corpses can be due to two distinct reasons either enhanced apoptosis or reduced engulfment. To distinguish between these two possibilities, I took advantage of the fact that apoptotic cell corpses in the germ line of adult hermaphrodites can be visualised with the vital dye acridine orange (Milstein and Hengartner, *unpublished*). While in wild-type hermaphrodites typically two apoptotic cell bodies are stained by acridine orange, there are none in mutations that affect the engulfment machinery (Milstein and Hengartner, *unpublished*). In a preliminarily experiment, I stained *rrf-3; tat-1(RNAi)* mutant worms with acridine orange and scored the number of fluorescent bodies in one arm of the adult hermaphrodite gonad. Interestingly, I observed almost equal numbers of fluorescent bodies in *rrf-3; tat-1(RNAi)* worms and in the control *rrf-3* hermaphrodites (data not shown), indicating that loss of *tat-1* function by RNAi rather results in decreased engulfment than increased apoptosis. However, 4D microscopy, which is currently being established in the lab by Lukas Neukomm, will allow the unequivocal discrimination between the two possibilities.

4.3. Discussion

PS exposure has been considered a hallmark of apoptotic cells in all multicellular organisms. Surprisingly, it has never been demonstrated whether apoptotic cells in *C. elegans* also expose PS. In this study, I provide strong evidence that PS is indeed exposed on apoptotic cells in *C. elegans*. This finding is of great interest, as it represents the basis for any further work that will investigate the mechanism of PS exposure and the putative role of PS as an 'eat-me' signal in *C. elegans*. Using an inducible GFP::AnxV reporter construct, I found that apoptotic cells in *C. elegans* expose PS, as demonstrated by the selective highlighting of dying cells in embryos and the germ line of adult hermaphrodites. GFP::AnxV staining is highly specific – only apoptotic or necrotic cells that have lost the asymmetry of phospholipids in their plasma membrane are labelled by GFP::AnxV. Consistently, PS exposure is impaired in *ced-3(n717)* or *ced-4(n1162)* mutant worms, implicating that PS exposure is dependent on apoptosis.

In mammals, PS is known to act as an 'eat-me' signal, which marks apoptotic cells for engulfment. Therefore, it is tempting to speculate that PS exposure on apoptotic cells in *C. elegans* serves the same purpose. This hypothesis is supported by the observation that ablating *tat-1* function by RNAi results in reduced PS exposure and impaired engulfment of apoptotic cell corpses in embryos and the adult hermaphrodite germ line. These data clearly demonstrate that efficient engulfment requires PS exposure. However, the engulfment defect, caused by impaired PS exposure, is only mild, suggesting that recognition and engulfment of apoptotic cell corpses does not only depend on PS as an 'eat-me' signal. Indeed, in mammals certain other, only weakly characterised 'eat-me' signals, such as Collectin-binding sites, TSP-1-binding sites, OxLDL-like sites and altered carbohydrates, have been found on the surface of apoptotic cells. But whether these molecules also play a role in the engulfment of apoptotic cell corpses in *C. elegans* remains to be determined.

ABCA1, a mammalian homologue of CED-7, has been reported to be required for PS exposure on the outer membrane leaflet of apoptotic cells. However, the number of GFP::AnxV labelled apoptotic cell corpses is not reduced in *ced-7* mutant worms, indicating that PS exposure is not affected. Although this finding is surprising given the published mammalian data, it is possible that ABCA1 and CED-7 have different substrate specificities. In general, ABC transporters are known to be able to transport a large variety of substrates, such as ions, sugars, vitamins, phospholipids, peptides and proteins. ABCA1 promotes the redistribution of phospholipids at the plasma membrane and is involved in reverse cholesterol transport. In fact, ABCA1 is mutated in patients affected with Tangier disease, a rare autosomal recessive disorder of lipid metabolism, characterised by impaired cholesterol efflux. While ABCA1 deficient mice display impaired clearance of apoptotic cell corpses, such a defect has not been reported in humans with reduced ABCA1 function. This disparity suggests that the function of ABCA1 might not be tightly conserved through evolution, explaining the different results for PS exposure obtained in ABCA1 or *ced-7* loss-of-function studies. Identifying the proper substrate(s) for CED-7 will shed some light on this issue.

Assuming a role for PS as an 'eat-me' signal in *C. elegans*, why did I not detect any engulfment defect in *psr-1* deficient worms? Although I did not confirm that the *psr-1* deletions completely disrupt PSR-1 expression by northern blot analysis, it is unlikely that both independently isolated *psr-1* mutations fail to eliminate *psr-1* function. Taking into account that a recent publication by Cui et al. (2004) reports nuclear localisation of mammalian PSR [23] and considering the circumstance that cell surface localisation of PSR has never been demonstrated directly, I postulate that the proposed function of *psr-1* as a PS receptor has to be revised. Analysis of the subcellular localisation pattern of PSR-1 would help to clarify its function in *C. elegans*. Resolving this ambiguity is of particular importance, especially as *psr-1* has been identified by homology to mammalian PSR.

By searching the literature for genes that might play a role in PS exposure on apoptotic cells in *C. elegans*, I came across the P-type ATPases,

which have been identified by close homology to the P-type ATPases found in yeast. Assuming that *C. elegans* ATPases might have the same function as their yeast homologues, one would expect that ablating their function by RNAi would impair PS translocation from the outer to the inner leaflet of the plasma membrane. Therefore, I did not expect to interfere with PS exposure on apoptotic cells upon RNAi treatment in *opIs117* transgenic worms. However, *tat-1(RNAi)* caused a strong reduction in the number of GFP::AnxV-labelled apoptotic cell corpses, suggesting that PS exposure is impaired in these animals. Moreover, the number of unengulfed cell corpses was increased in embryos and in the germ line of *rrf-3; tat-1(RNAi)* worms.

tat-1 encodes two isoforms, Y49E10.11a and Y49E10.11b, differing by the last exon, which is missing in variant b. Compared to other genes *C. elegans tat-1* is rather a large gene, covering a genomic region of more than 21kb on the right arm of chromosome III. It consists of 17 mainly short exons that give rise to a transcript of 3.4kb in size. Beside the four P-type ATPase consensus sequences, TAT-1 has a predicted haloacid dehalogenase-like dehydrolase domain (residues aa382-786) and a predicted cytochrome *c* heme-binding site. Consistent with its predicted function as a P-type ATPase TAT-1 contains 10 predicted transmembrane domains. Based on sequence similarity five other genes have been grouped into the same P-type ATPase subfamily with *tat-1*. However, the function of none of them has been analysed yet.

How can the loss of *tat-1* function cause a defect in PS exposure? There are two possible explanations. Firstly, TAT-1 might function as a floppase, i.e. an outwardly directed, ATP-dependent transporter that becomes activated in the dying cell, leading to PS exposure. However, this model contradicts the proposed function of a P-type ATPase, as it has been deduced from multiple studies in yeast and mammals. The alternative model comprises the assumption that TAT-1 displays two different functions: I) in healthy cells, TAT-1 might act as a translocase, promoting the inwardly directed, ATP-dependent transport of PS across the plasma membrane; II) in dying cells, TAT-1 undergoes a conformational change, possibly through *ced-3*-

dependent cleavage, that enables it to activate a scramblase or floppase activity, resulting in PS exposure. Since *tat-1(RNAi)* does not seem to cause any defect in healthy cells, one can assume that the flippase activity of TAT-1 is redundant with another, yet unidentified, protein. Although the second model is mainly based on speculations, it convinces through its consistence with other data.

Based on homology, TAT-1 is most similar to yeast Drs2p, a protein with proposed translocase activity (Supplementary Figure 3). Drs2p belongs to a conserved family of P-type ATPases, including Dnf1p, Dnf2p, Dnf3p, and Neo1p that have overlapping functions in vivo. Drs2p localises to the late Golgi membrane and is involved in protein transport from the TGN to the plasma membrane. Besides its role in protein trafficking, Drs2p has been shown to mediate translocation of fluorescently-labelled PS from the luminal to the cytoplasmic leaflet of the TGN.

By analogy with the proposed function of DRS2, *tat-1* could also be involved in protein trafficking between the TGN and the plasma membrane. Therefore, compromising the function of *tat-1* might compromise vesicle trafficking and proper protein sorting from the TGN to the plasma membrane. Thus, abolishing the function of *tat-1* by RNAi might affect the proper localisation of a scramblase in the plasma membrane, which might be required for PS exposure at the cell surface upon apoptosis. By today's state of knowledge, I cannot exclude this possibility. However, in *tat-1(RNAi)* worms protein trafficking seems not to be generally affected, as GFP::AnxV is still properly secreted and the fusion protein CED-1::GFP is properly localised around apoptotic cells in the germ line (data not shown).

The closest mammalian homologue of *tat-1* is the gene *1a*, which has originally been cloned from bovine chromaffin granules (ATPase II) (Supplementary Figure 3). Although there is some evidence that *1a* encodes a PS translocase, knock down of *1a* function by RNAi did not result in reduced PS transport in mammalian cells (P. Williamson, *unpublished*). However, it has not been tested whether ablating the function of *1a* compromises PS

exposure in cells undergoing apoptosis. Importantly, the gene *1b*, which shows highest homology to *1a*, has not been tested yet for a role in PS exposure. It could be possible that in mammalian cells, the gene *1a* encodes the vesicle transporter and the gene *1b* encodes the plasma membrane transporter. In this case, *C. elegans* TAT-1 might act as both, a vesicle and a plasma membrane transporter.

Several questions concerning the role of *tat-1* in PS exposure still await an answer. What is the expression pattern of *tat-1*? Where in the cell does TAT-1 localise? To address this questions, I tried to build a *tat-1::GFP* minigene; however, I was not able to amplify the *tat-1* promoter by PCR. This failure might be due to the large amount of highly repetitive sequences in the promoter region of *tat-1*. Similarly, I could not generate a *tat-1::GFP* reporter construct to analyse the subcellular localisation pattern of TAT-1. It appears that *tat-1* expression is toxic to bacterial cells, possibly due to its properties of a transmembrane protein. It might be possible that the generated plasmid contains a hidden promoter sequence, which would allow *tat-1::GFP* expression in bacteria, a circumstance that would explain the observed lethality. To avoid these problems and to finally resolve the subcellular localisation pattern of *tat-1*, antibodies against TAT-1 are being generated. Knowledge of the subcellular localisation pattern of TAT-1 will provide a good starting point to decide between the proposed functions of TAT-1 mentioned above.

4.4. Material and Methods

Mutations and Strains

Methods for culturing *C. elegans* strains have been described previously [47]. All strains were grown at 20°C. All mutants used in this study were derived from the wild-type variety Bristol strain N2. The following mutations are described in Riddle et al. (1997)[48], except *ced-12(oz167)* [20], *rrf-3(pk1426)* [46] and *gla-1(op234)* (Milstein and Hengartner, *unpublished*) which have been described elsewhere. LGI: *ced-1(e1735)*, *ced-12(oz167)*, *gla-1(op234)*. LGII: *rrf-3(pk1426)*. LGIII: *ced-4(n1162)*, *ced-6(n1813)*, *ced-7(n1996)*, *ced-7 (n1892)*, *ced-9(n1653ts), unc-119(ed3)*. LGIV: *ced-2(e1752)*, *ced-3(n717)*, *ced-5(n1812)*, *ced-10(n1993)*, *psr-1(tm469)*, *psr-1(ok714)*. LGX: *ced-8 (n1891)*, *mec-4(u231)*. Deletion alleles of *psr-1* were detected by PCR using the following primer sequences: *tm469* 5'-cagagtttttggacgatatgaagg-3' and 5'-gcatttaacaaaaatacatcgtgtc-3', *ok714* 5'-gacagtttgtcgtttgccac-3' and 5'-cgcatcaagaggaacaaaca-3'.

Plasmid Constructs and Generation of Transgenic Animals

The cDNA of human Annexin V was obtained from C. Reutelingsperger (pEGFP-C1-AnxV) and a construct containing the *sel-1* signal sequence (BBC1) was obtained from I. Greenwald (Grant and Greenwald, 1997). Both sequences were amplified by PCR and tagged with the following restriction sites: *sel-1*: 5'Asc I, 3'Asc I; Annexin V: 5'Asc I, Not I, 3'Fse I. GFP was amplified from the Fire vector pPD117 (Fire vectors, 1997) and tagged on both ends with the restriction site Not I. The tagged fragments were subcloned into the modified heat shock vector p114Q, creating the inducible pSZ8 expression vector [$P_{hsp16.41}$*::sel-1::GFP::AnxV*]. The control construct pSZ12 [$P_{hsp16.41}$*::sel-1::GFP*] was built by subcloning the *sel-1* Asc I cassette into p114Q. GFP was amplified from pPD117 and tagged with the restriction sites Asc I and Fse I and subsequently cloned into the p114Q vector containing the Asc I *sel-1* cassette. Low copy transgenic animals were generated as previously described [49]. The protocol was adapted as follows: *unc-119(ed3)* mutant worms were grown in liquid culture. pSZ8 and pSZ12 were

precipitated with pPDMM016 [*unc-119(+)*], a gift from J. Austin, onto gold beads in a ratio of approximately 6:1 and shot at *unc-119(ed3)* mutant worms using a Biolistic PDS-1000 bombardment apparatus (Bio-Rad). Bombarded worms were washed onto multiple seeded large plates and allowed to starve. Starved worms were chunked onto fresh seeded plates and screened for *unc–119(+)* transgenic animals. Rescued worms that expressed GFP after heat shock were kept and analysed. At least two integrated lines with similar expression patterns were generated for each construct. All further experiments were done with the integrants *opIs117* [$P_{hsp16.41}$*::sel-1 ::GFP::AnxV*] and *opIs172* [$P_{hsp16.41}$*::sel-1:: GFP*].

RNAi expression vectors of the five predicted *C. elegans* aminophospholipid transporters were obtained from N. Lyssenko. The *tat-1* RNAi expression vector was built by subcloning a 0.8kb Spe I, Hind III fragment from the *C. elegans* EST yk34c11 into pPD129.3. A second *tat-1* RNAi construct, pSZ3, was built by amplifying a ~0.5kb, Asc I, Fse I tagged fragment from the *tat-1* genomic locus. The primers were as follows: 5'-cataggcgcgcccacagaggcaagagataataatcg-3' and 5'-cataggccggccgtcattgttgaa gtgatatccagc-3'. The Asc I, Fse I tagged *tat-1* fragment was subcloned into pLB4440 and transformed into the *E. coli* strain HT115. RNAi expression vectors of the other aminophospholipid transporters were built by subcloning a fragment from the corresponding EST into pPD129.3. *tat-2*: 1.6kb Not I, Xho I fragment of EST yk209d5; *tat-3*: ~1.1kb Bgl II, Pst I fragment of EST yk395E5; *tat-4*: 0.9kb Xba I of EST yk126b10; *tat-5*: 0.7kb Spe I Hpa I fragment of EST yk23e10. All other RNAi vectors used in this study were kindly provided by J. Ahringer.

Cell Corpse Assays

Worms were mounted on 4% agarose pads in a drop of M9 buffer containing 30mM NaN_3 or 3-5mM levimasole and observed by microscopy using a Leica DMRA microscope equipped with standard epifluorescence and appropriate filter sets for visualising GFP, Hoechst 33342, and SYTO41. Images were taken using an Orca ER camera using Openlab software.

Pictures were false coloured and further edited in Adobe Photoshop 7.0. To analyse GFP staining in the gonad of adult *opIs117* or *opIs172* hermaphrodites, worms were staged as L1s by hypochlorite treatment and heat shocked 72 hours later as adults at 33°C for 30 min. Six hours after heat shock, GFP::AnxV-labelled cell corpses were scored in one gonad arm using epifluorescence. Apoptotic cell corpses were identified as highly refractile disks in the germ line of identical treated hermaphrodites using Nomarski optics.

Immunofluorescence Staining

To visualise the condensed DNA of apoptotic cells, worms were stained with 2mg/ml Hoechst33342 (Molecular Probes) in dH_2O with some OP50 bacteria for 1 hour at room temperature. After washing once with dH_2O, the worms were transferred to a seeded NGM agar plate and allowed to destain for ~1 hour in order to reduce background staining in the intestine. To visualise engulfed cell corpses, worms were stained with 50μM SYTO41, following the same protocol as described above. Apoptotic cell corpses in the germ line of *rrf–3* mutant worms were stained with acridine orange (AO) by adding 500μl of M9 buffer containing AO (0.02mg/ml, Molecular Probes) to a small seeded plate. Worms were kept for 1 hour in the dark, were then washed to a fresh seeded plate and allowed to destain for 1 hour in the dark.

RNAi Experiments

RNAi experiments were done as described in Fraser et al. (2000)[50]. About 30-50 synchronised L1s were placed on NGM agarose plates seeded with *E. coli* producing double-stranded RNA (dsRNA). Worms were grown for 3 days, then heat shocked at 33°C for 30 min. 6 hours after heat shock the effect of RNAi on PS exposure was assessed by fluorescent and DIC microscopy.

Time Course Analysis

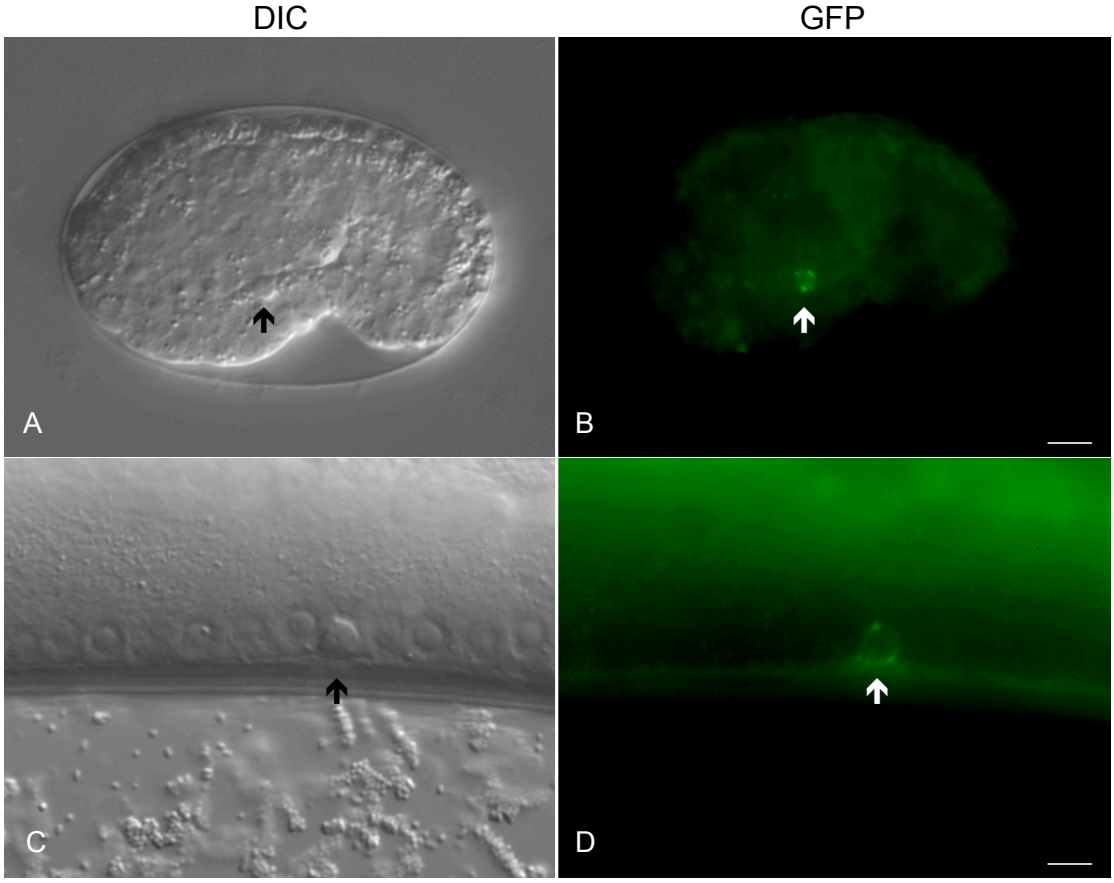
To study the effect of *tat-1(RNAi)* on PS exposure more precisely, RNAi treated animals were assessed for GFP::AnxV labelling of apoptotic cell

corpses every 12 hours, starting at the L4/adult molt. Simultaneously, the number of apoptotic germ cells was scored in animals derived from the same population. RNAi and heat shock were carried out as described above.

To avoid heat shocking the same worms multiple times, worms were grown simultaneously on several RNAi plates that were heat shocked only once, i.e. 6 hours before scoring.

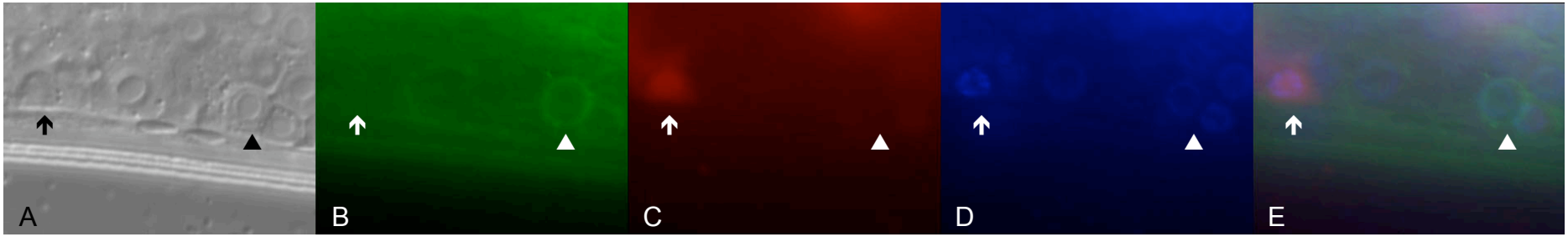
4.5. Figures and Tables

Figure 1: GFP::AnxV Labels Apoptotic Cell Corpses in Embryos and in the Adult Hermaphrodite Germ Line



Transgenic *opIs117* [*P* $_{hsp16.41}$*::sel-1::GFP::AnxV*] worms were heat shocked and analysed as described in Materials and Methods. GFP::AnxV labels both embryonic cell corpses (A and B, arrow) as well as apoptotic cell corpses in the adult hermaphrodite germ line (C and D, arrow). Size bar, 5μm.

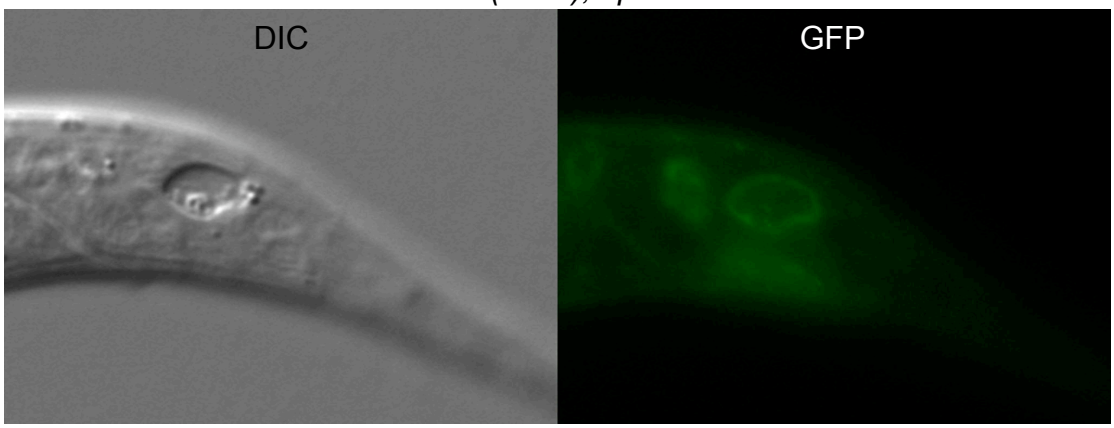
Figure 2: GFP::AnxV Stains Early Apoptotic Cell Corpses in the Adult Hermaphrodite Germ Line



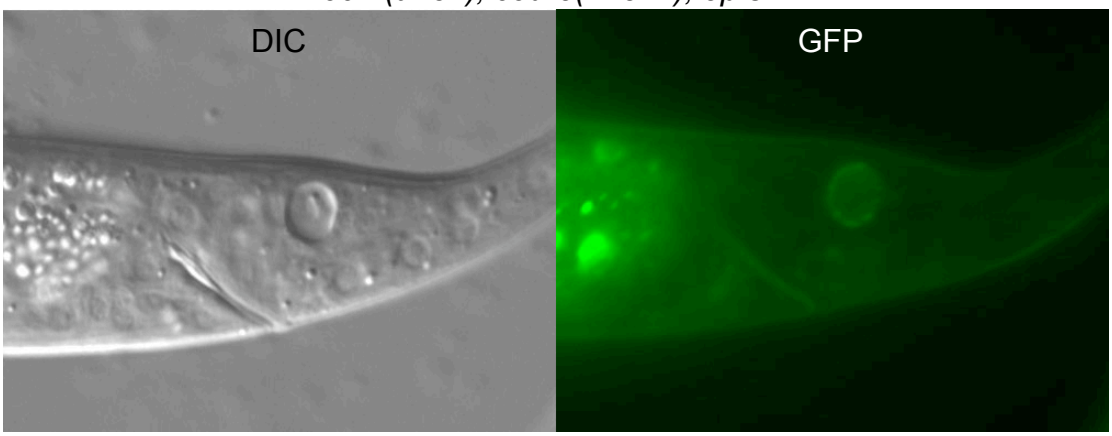
Heat shocked *gla-1(op324)*; *opIs117* adult hermaphrodites were co-stained with SYTO41 and Hoechst 33342 to determine the apoptotic stage of GFP::AnxV labelled germ cell corpses. In the DIC picture (A) two apoptotic cell corpses can be distinguished: an early apoptotic corpse that does not yet have the characteristic appearance of a refractile disk (arrowhead) and a late corpse with typical apoptotic morphology (arrow). The early apoptotic cell corpse is marked by GFP::AnxV (B, arrowhead); this corpse is not engulfed yet, as confirmed by the absence of SYTO41 (C, arrowhead) and weak Hoechst 33342 staining (D, arrowhead). In contrast, the late, engulfed cell corpse (C and D, arrow) is not labelled with GFP::AnxV.

Figure 3: GFP::AnxV Stains Necrotic Cell Corpses

mec-4(u231); opIs117

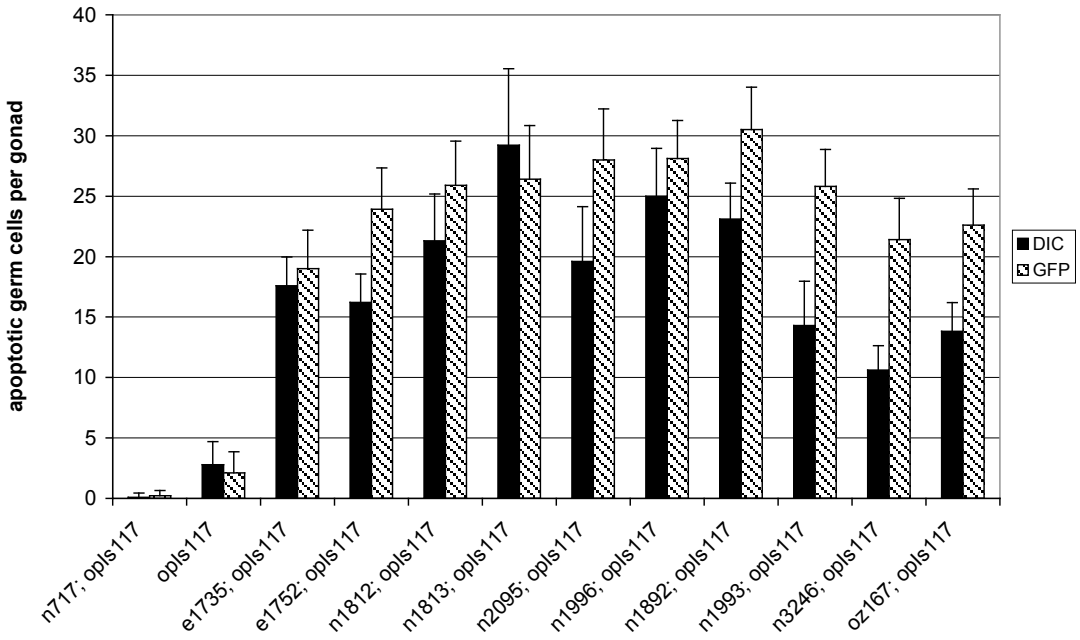


mec-4(u231); ced-5(n1812); opIs117



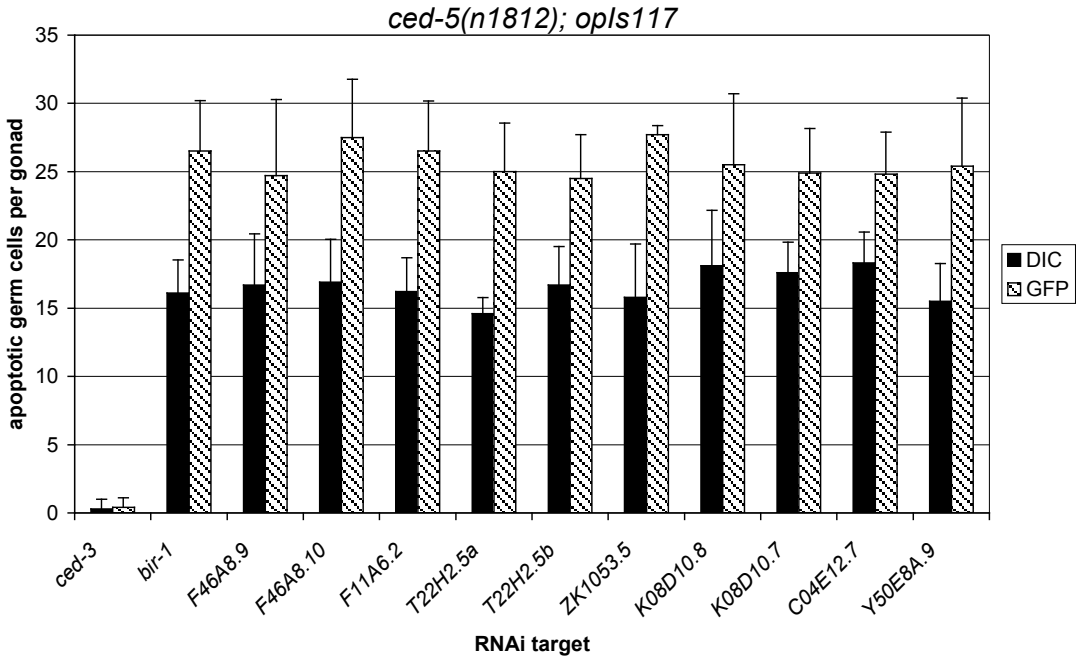
The dominant mutation *mec-4(u231)* causes necrotic cell death of the six touch-receptor neurons in the larval tail. A mixed stage population of *mec-4 (u231); opIs117* or *mec-4(u231); ced-5(n1812); opIs117* mutant worms were heat shocked as described in Materials and Methods. Six hours after heatshock young L1s were picked and examined for necrotic cell corpses using Nomarski and GFP epifluorescence microscopy.

Figure 4: The Known Engulfment Genes Are Not Required For PS Exposure



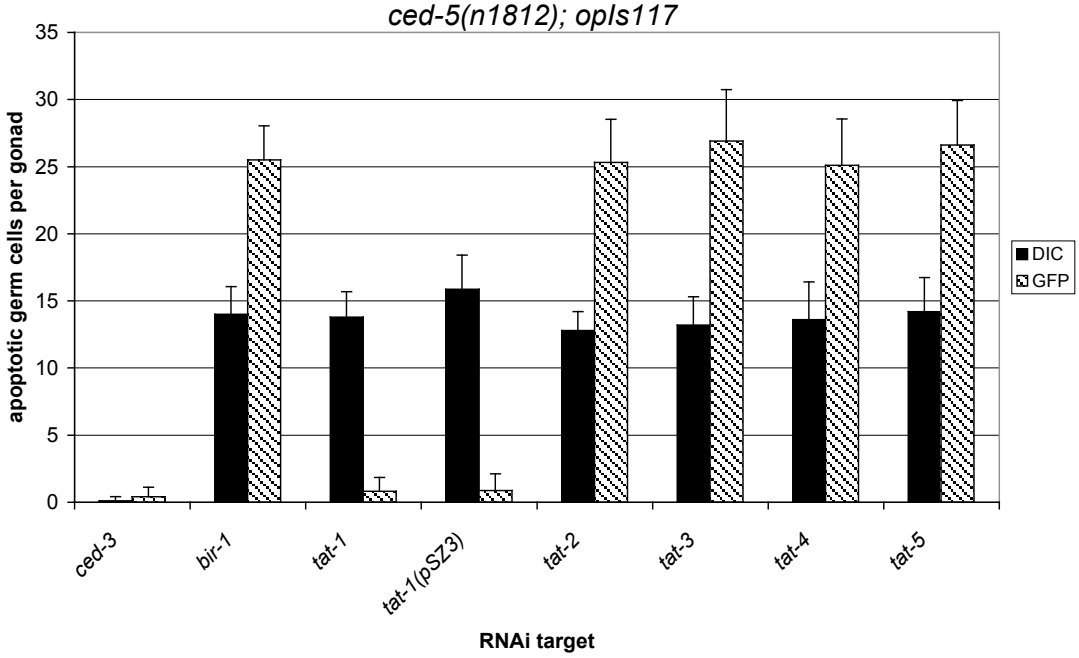
Germ cell corpses were counted 24 hours after the L4/adult molt using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=10.

Figure 5: *C. elegans* Scramblases Are Likely Not Involved in PS Exposure



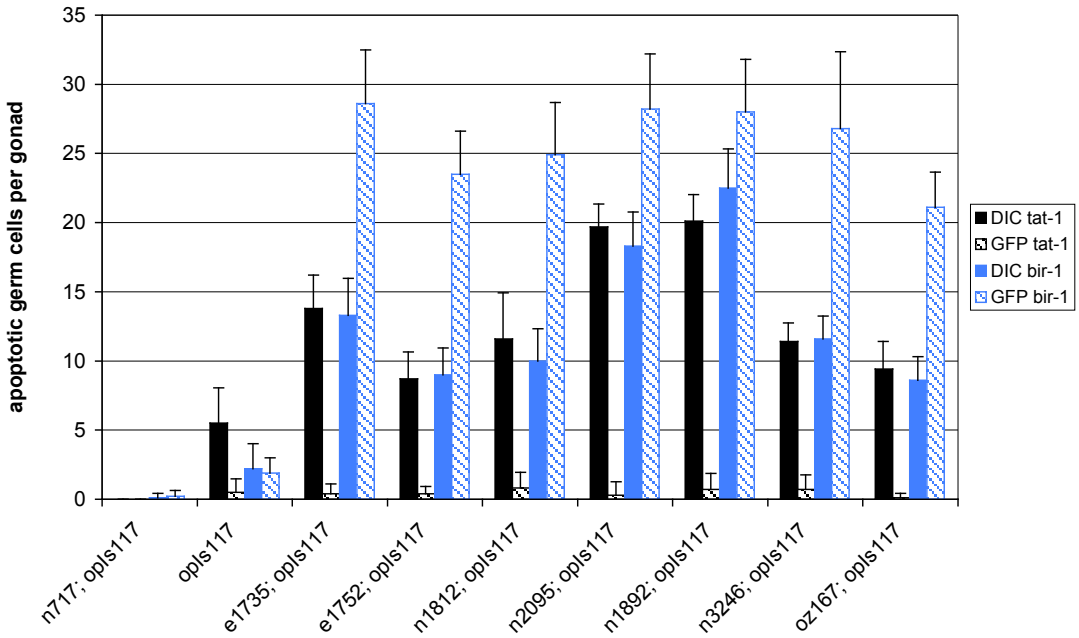
ced-5(n1812); opIs117 worms were staged and put as L1s onto 2mM IPTG plates seeded with bacteria expressing the appropriate RNAi construct. Germ cell corpses were counted 24 hours after the L4/adult molt using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=10.

Figure 6: The Translocase *tat-1* Is Required for PS Exposure



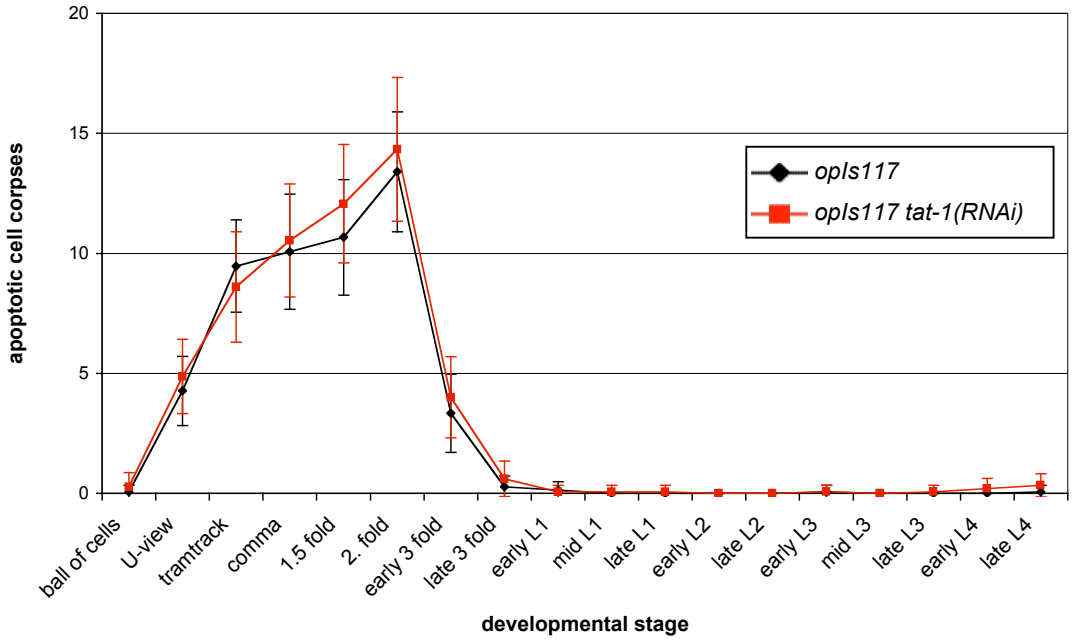
ced-5(n1812); opIs117 worms were staged and put as L1s onto 2mM IPTG plates seeded with the appropriate RNAi expressing bacteria. Germ cell corpses were counted 24 hours after the L4/adult molt using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=10.

Figure 7: *tat-1(RNAi)* Impairs PS Exposure in All Engulfment Mutants



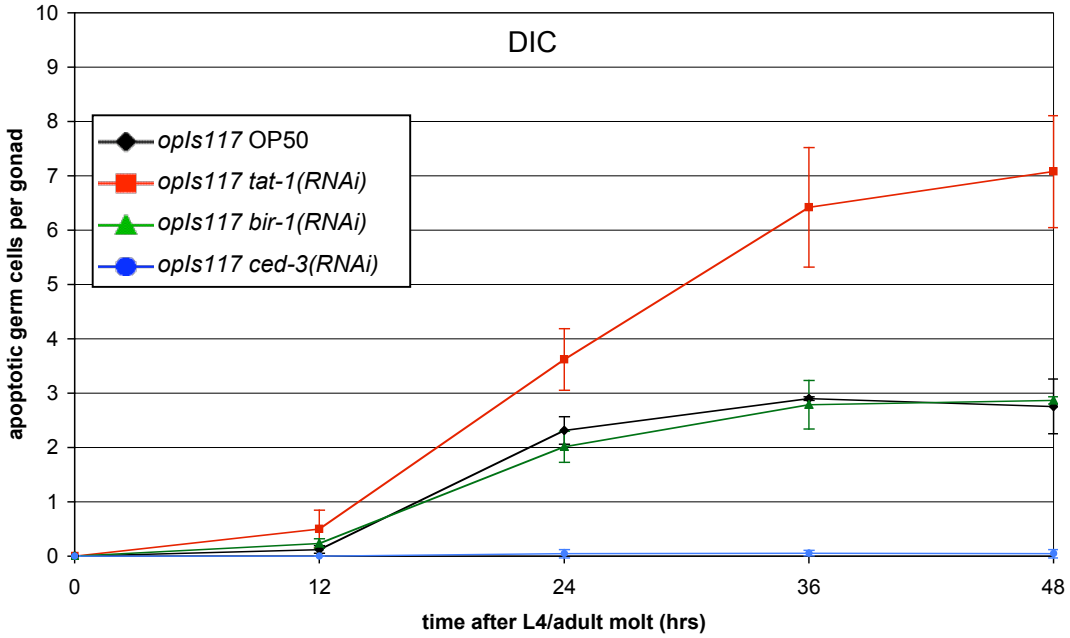
The different engulfment mutants scored in this experiment were synchronised by hypochlorite treatment and put as L1s onto 2mM IPTG RNAi plates seeded with bacteria expressing the appropriate RNAi construct. Germ cell corpses were counted 24 hours after the L4/adult molt using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=10.

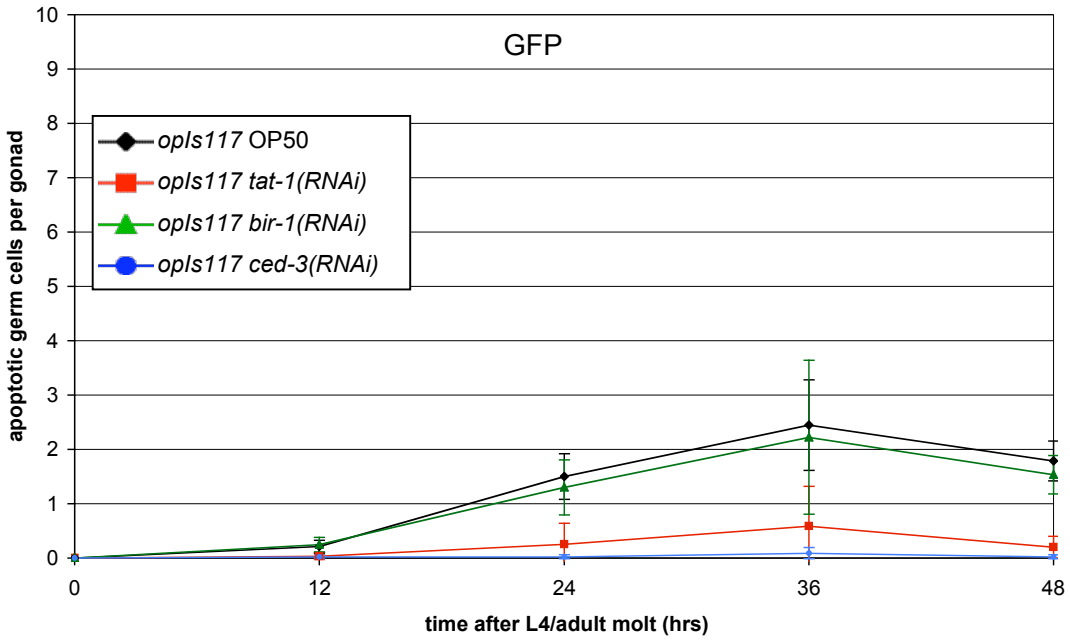
Figure 8: The Number of Apoptotic Cell Corpses Is Not Significantly Enhanced in *tat-1(RNAi)* Embryos



opIs117 worms were synchronised by hypochlorite treatment and put as L1s either onto standard NGM agar plates or 2mM IPTG *tat-1* RNAi plates. Embryos of the F1 generation were picked and analysed by Nomarski microscopy. Apoptotic cell corpses were scored in whole embryos till the two fold stage and from the early three fold stage onward only in the head region (n=15).

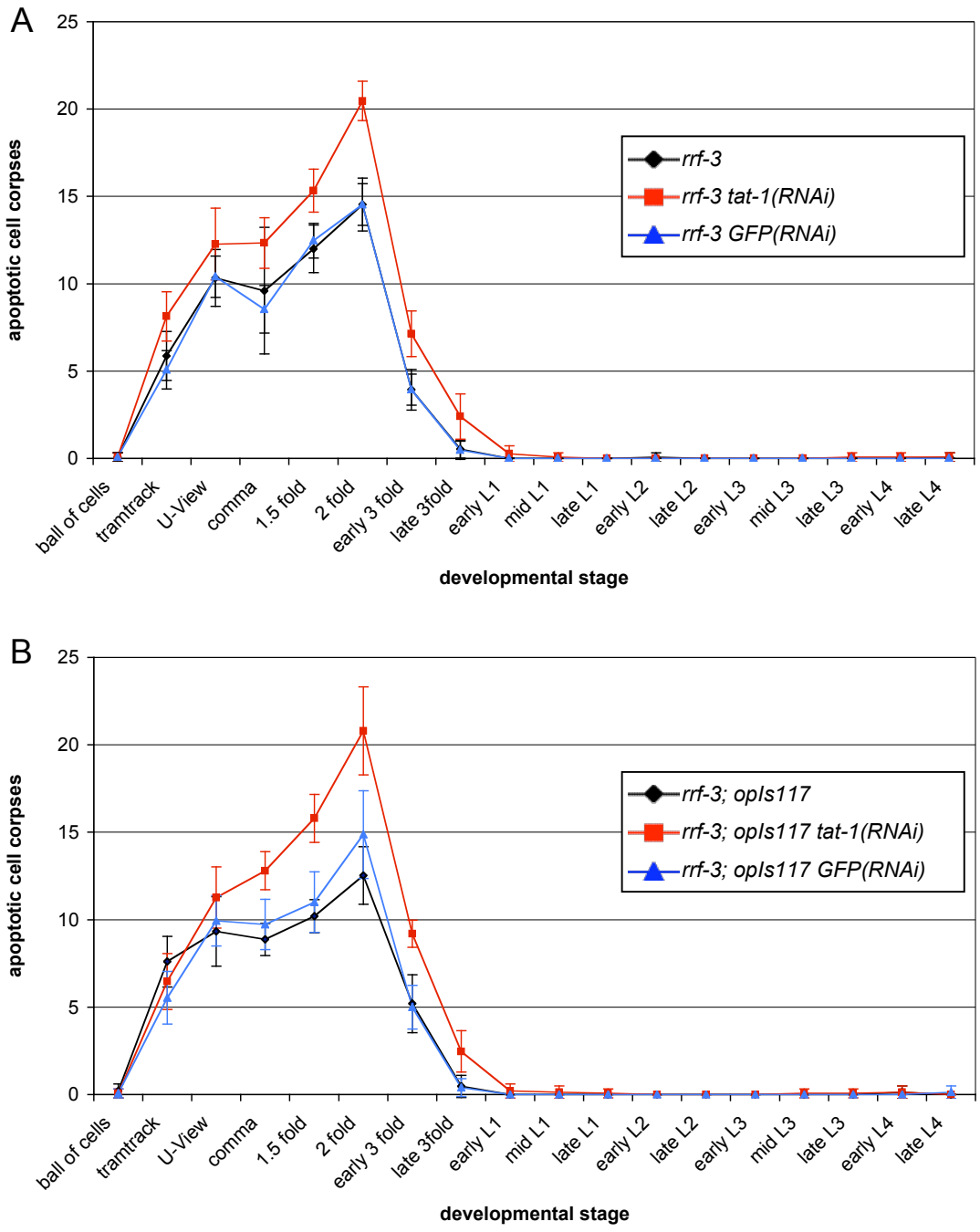
Figure 9: *tat-1(RNAi)* Increases the Number of Apoptotic Cell Corpses in the Germ Line of *opIs117* Hermaphrodites





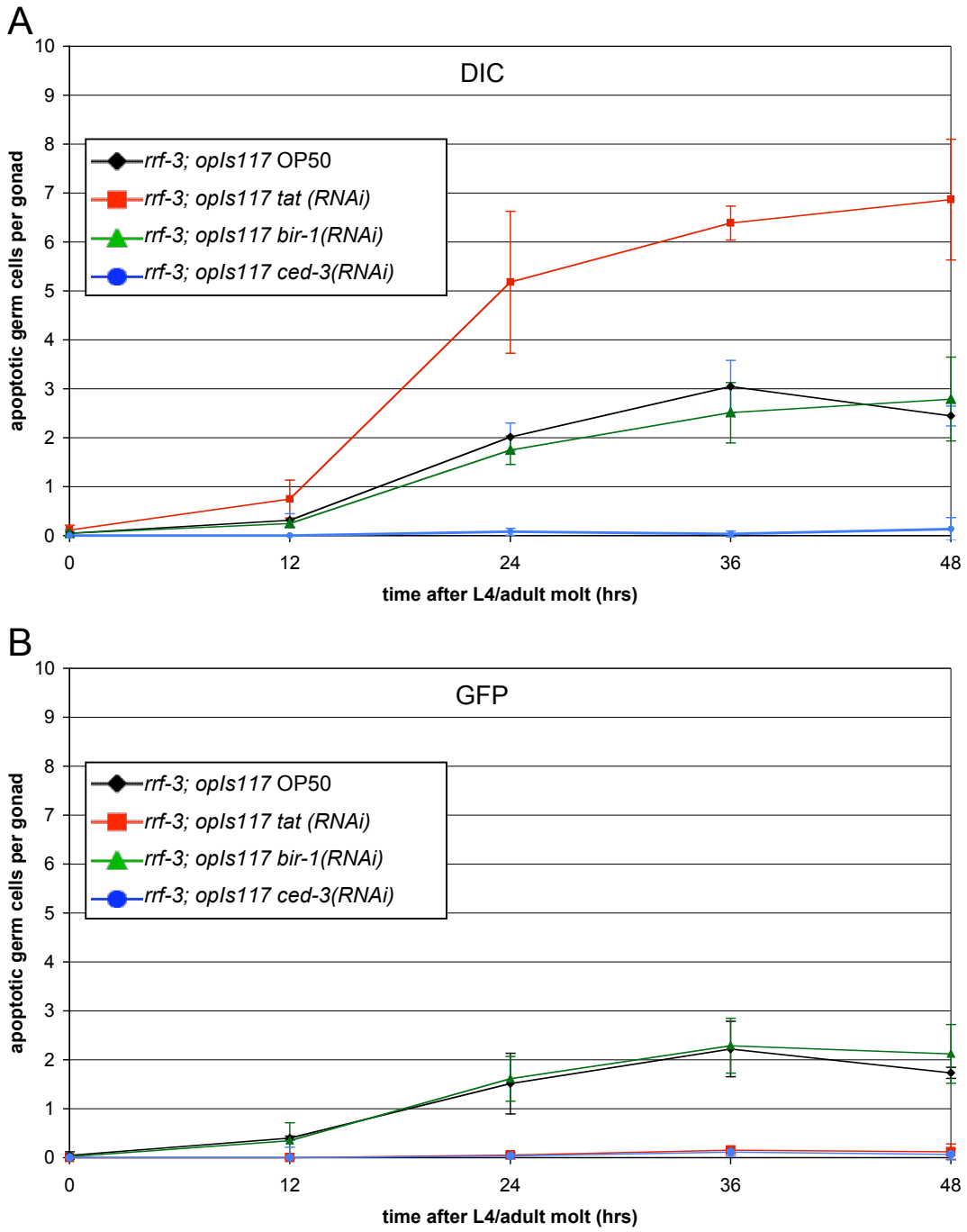
opIs117 worms were synchronised by hypochlorite treatment and put as L1s either onto standard NGM agar plates or 2mM IPTG RNAi plates. Time course analysis of apoptotic germ cells was started at the L4/adult molt (0hrs) using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=15.

Figure 10: The Number of Apoptotic Cell Corpses Is Significantly Enhanced in *rrf-3; tat-1(RNAi)* Embryos



rrf-3 (A) or *rrf-3; opIs117* (B) worms were synchronised by hypochlorite treatment and put as L1s either onto standard seeded NGM agar plates or 2mM IPTG *tat-1* RNAi plates. Embryos of the F1 generation were picked and analysed by Nomarski microscopy. Apoptotic cell corpses were scored in whole embryos till the two fold stage and from the early three fold stage onward only in the head region (n=15).

Figure 11: *tat-1(RNAi)* Significantly Increases the Number of Apoptotic Cell Corpses in the Germ Line of *rrf-3* Hermaphrodites



rrf-3; opIs117 worms were synchronised by hypochlorite treatment and put as L1s either onto standard NGM agar plates or 2mM IPTG RNAi plates. Time course analysis of apoptotic germ cells was started at the L4/adult molt (0hrs) using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=15.

Table 1: GFP::AnxV Staining Is Dependent on Apoptosis

	apoptotic germ cells per gonad	
Genotype	DIC	GFP
opIs117[P_{hsp}::sel-1::GFP::AnxV]	2.8±1.9	2.1±1.7
opIs172[P_{hsp}::sel-1::GFP]	2.7±0.4	0
ced-3(n717); opIs117[P_{hsp}::sel-1::GFP::AnxV]	0.1±0.3	0.2±0.4
ced-4(n1162); opIs117[P_{hsp}::sel-1::GFP::AnxV]	0	0.1±0.3
ced-9(n1653); opIs117[P_{hsp}::sel-1::GFP::AnxV]	14.6±3.0	11.4±1.9
gla-1(op234); opIs117[P_{hsp}::sel-1::GFP::AnxV]	14.9±2.7	12.1±2.6
ced-3(n717); ced-5(n1812); opIs117[P_{hsp}::sel-1::GFP::AnxV]	0.1±0.3	0.4±1.0

Germ cell corpses were counted 24 hours after the L4/adult molt using Nomarski optics (DIC) and GFP epifluorescence. For each animal observed only one gonad arm was scored. All data are averages ± standard deviation (SD), n=10.

Table 2: GFP::AnxV Stains Vacuolated PML Cell Corpses

	DIC	GFP
mec-4(u231); ced-5(n1812); opIs117	vacuolated PML cells per animals	
OP50	42/50	26/50
ced-3(RNAi)	44/50	29/50
tat-1(RNAi)	46/50	30/50

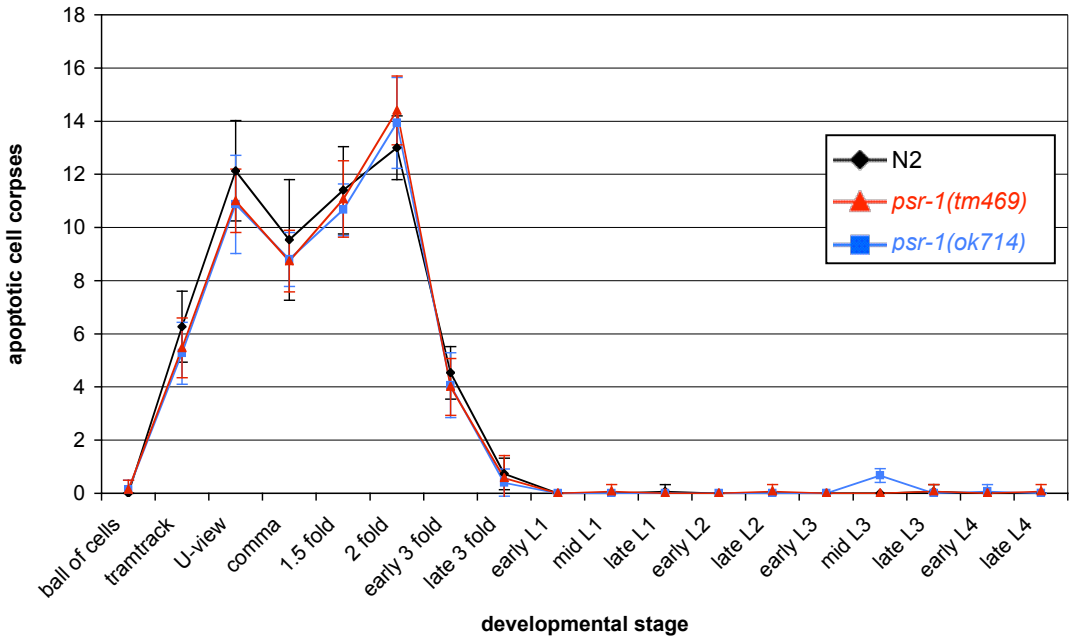
A mixed stage population of *mec-4(u231); ced-5(n1812); opIs117* mutant worms was heat shocked as described in Materials and Methods. Five hours after heatshock worms were washed off the plate with M9 buffer and embryos were allowed to hatch on the plate. One hour later young L1s were picked and screened for vacuolated PML cell corpses in the tail using Normarski (DIC) and epifluorescence microscopy. Data are averages from two different experiments.

Table 3: *C. elegans t*ransbilayer *a*mphipath *t*ransporters

Gene		class
tat-1	Y49E10.11	I
tat-2	H06H21.10	I
tat-3	W09D10.2	II
tat-4	T24H7.5	II
tat-5	F36H2.1	IV
tat-6	F02C9	IV

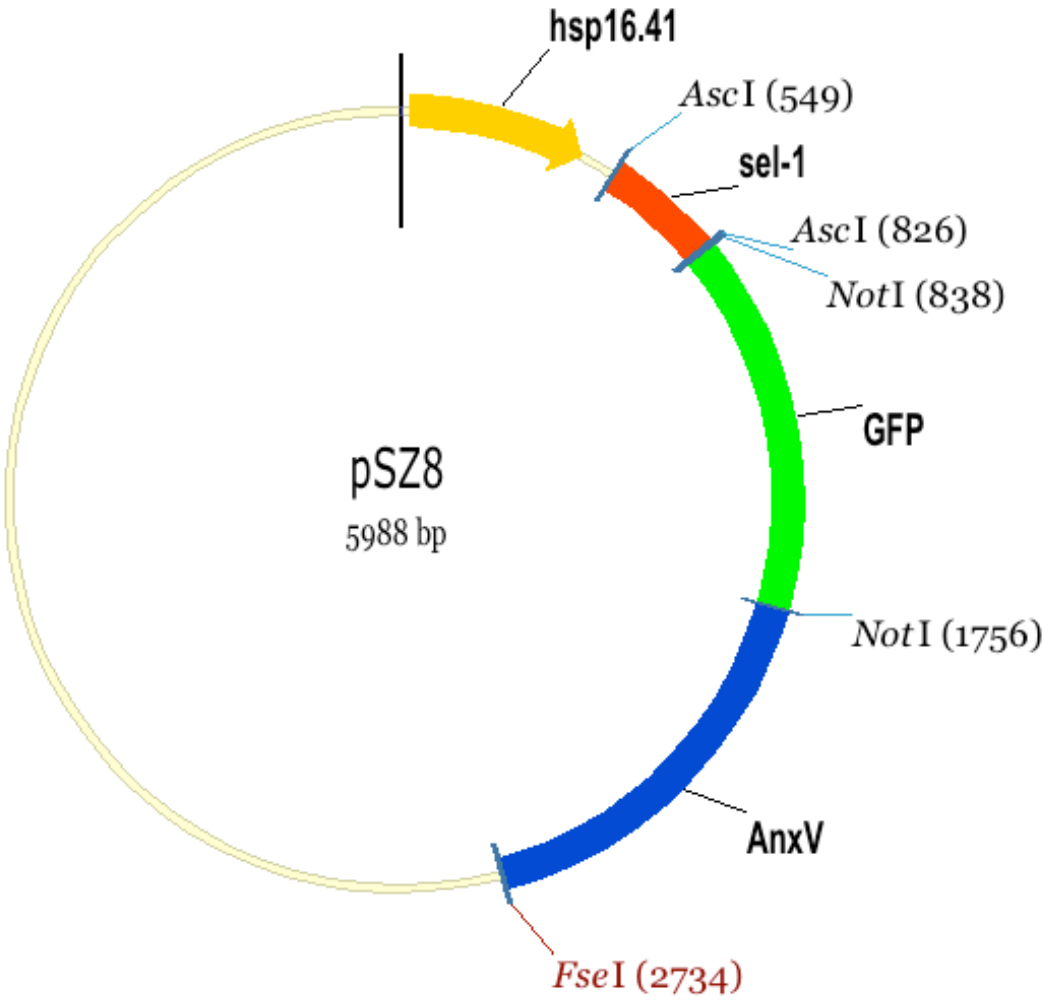
The six potential *C. elegans* translocases have been grouped into three classes based on their homology to the translocase family members of other species. See also Supplementary Figure 3.

Supplementary Figure 1: *psr-1* Loss-of-Function Does Not Cause an Engulfment Defect in *C. elegans*



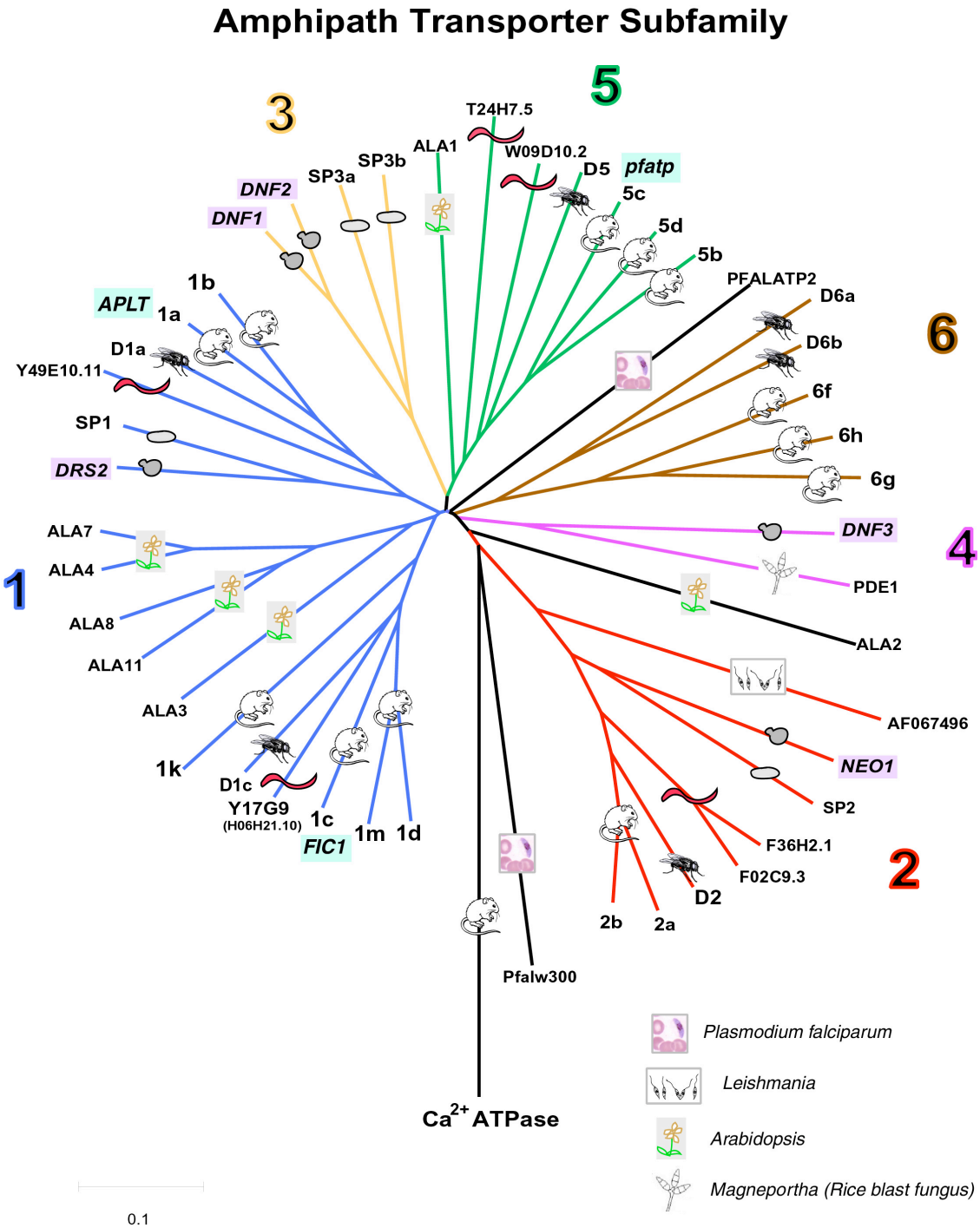
To precisely follow the persistence of apoptotic cell corpses over time, the embryonic and larval stages defined by Sulston et al. (1983) have been divided into 18 subclasses, which can be distinguished by Nomarski optics. Persistent cell corpses were scored in whole embryos at early developmental stages and from the early three fold stage onward in the head region (n=15). All data are averages ± standard deviation (SD).

Supplementary Figure 2: Schematic Representation of the GFP::AnxV Reporter Construct



The vector backbone contains an *unc-54* 3'UTR sequence and an ampicillin resistance gene (not shown).

Supplementary Figure 3: Dendogram of the Transbilayer Amphipath Transporter Family



Courtesy of Peggy Halleck

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Chapter 5

Future Directions

5.1 Genetic Elucidation of the Engulfment Machinery

Since the late 1980's significant progress has been made in deciphering the molecular mechanisms that lead to the recognition and engulfment of apoptotic cell corpses in *C. elegans*. Seven genes – *ced-1*, *-2*, *-5*, *-6*, *-7*, *-10*, and *-12*, have been identified that are required for the efficient engulfment of apoptotic cells [1-3]. Loss-of-function mutations in any of the engulfment genes interfere with the normal function of the phagocytic machinery and cause the persistence of many unengulfed cell corpses in the worm. However, none of these genes appears to be absolutely essential, as many apoptotic cell corpses are still properly removed in the different engulfment mutants. Double mutant analyses and overexpression studies grouped the known engulfment genes into two distinct, partially redundant pathways that seem to converge at the level of CED-10, and subsequent biochemical studies unravelled the molecular function of some, but not all of these genes. Despite substantial progress, many open questions remain concerning the various genetic and molecular characteristics of the engulfment machinery.

Far from being understood are the interactions between the apoptotic and the engulfing cell. How does the dying cell mark itself as being apoptotic and ready for engulfment? What kind of cell surface changes occur on the dying cell? How does the engulfing cell recognise the apoptotic cell? So far, PS represents the best candidate for a so-called 'eat-me' signal that becomes exposed on the surface of apoptotic cells. However, the moderate engulfment defect observed in worms with impaired PS exposure suggests that further molecules play a role in the tight interplay between the apoptotic and the engulfing cell. In addition, it remains unclear how exposed PS is recognised by the engulfing cell. Recent studies, published by Böse and colleagues (2004) and my personal data, indicate that the well-known PS receptor does not play a role in the removal of apoptotic cells either in mammals or in worms [4,5]. These provoking results entail several important questions: Is there an as-yet unidentified PS receptor that is required for the primary recognition and phagocytosis of apoptotic cells? Or is PS bound by so-called bridging

molecules that mediate the recognition and engulfment of the apoptotic cell through interaction with their cognate receptors? In *C. elegans*, proteins with a bridging function have not been described so far, and the worm homologues of mammalian bridging molecules do not appear to play a role in the engulfment of apoptotic cells, as observed with the predicted homologue of mammalian MFG-E8 (S. Charette and M.O. Hengartner, *personal communication*).

The transmembrane protein CED-1 is believed to function as a receptor for the engulfment signal(s) provided by the dying cell. However, its cognate ligand has not been identified yet. Since the appropriate mammalian homologue of CED-1 is still under debate, one can only speculate on potential ligands. One of the potential homologues of CED-1 is the mammalian CD91/LRP that has been shown to bind the bridging molecule calreticulin [6,7]. However, in *C. elegans* neither calreticulin nor calnexin are required for the engulfment of apoptotic cells (G. Lettre and S. Züllig, *unpublished*). Others have grouped CED-1 into the family of scavenger receptors, which have been shown to recognise lipoproteins and anionic phospholipids [8]. Thus, although no direct evidence exists, it might be possible that CED-1 binds PS. Human MEGF-10, a member of the multiple epidermal growth factor domain-like protein family, has also been proposed to be a candidate orthologue of CED-1 [9]. Hence, CED-1 might instead interact with proteins containing EGF-domains. Further studies are definitively required to elucidate the specific interactions between the apoptotic and the engulfing cell.

The upstream signalling events in the engulfment pathway defined by *ced-2*, *-5, -10*, and *-12*, are currently unknown. It is not clear, how the apoptotic cell is recognised, as the receptor that signals to this signalling cassette has not been identified yet. It might be possible that more than one receptor signals to CED-2 and that these receptors function redundantly. Alternatively, the responsible receptor could be essential for viability and ablating its function would result in lethality. In mammalian cells, it has been found that CrkII/CED-2 binds the integrin receptor $\alpha_v\beta_5$ via $p130^{cas}$; however, in the *C. elegans* genome no bona-fide homologue of $p130^{cas}$ has been

identified [10]. Furthermore, the integrin receptors do not appear to be involved in the engulfment of apoptotic cell corpses in the worm [11]. Hence, *C. elegans* might diverge from mammalian cells at the recognition step, or may involve an as yet unidentified receptor complex to mediate recognition of the apoptotic cell.

Signalling components that act downstream of the small GTPase CED–10 in the engulfment of apoptotic cells in *C. elegans* are also unknown. Although the worm homologues of the Arp2/3 protein complex have been reported to play an essential role in cell migration during ventral closure, they apparently do not participate in the phagocytosis of apoptotic cells[12]. In addition, mutations of the GEX-2 and GEX-3 proteins, which are highly conserved with vertebrate homologues implicated in binding the small GTPase Rac, result in strong defects in morphogenesis and cell migrations, but show normal engulfment [13]. As these results demonstrate, interfering with the actin cytoskeleton can result in strong morphogenetic defects that might cause embryonic lethality. Thus, proteins involved in the reorganisation of the cytoskeleton could have been missed in conventional engulfment screens, since their function would be essential during embryonic development. Genetic screens that allow the recovery of homozygous lethal mutants, e.g. a screen for temperature-sensitive engulfment mutants, might overcome this problem. In addition, modifier screens that enhance or suppress the engulfment defect caused by partial *ced-10* loss-of-function mutations could facilitate the identification of further engulfment genes. Alternatively, downstream effectors of CED-10 could be identified by screening for suppressors of the embryonic lethal phenotype conferred by a strong *ced-10* loss-of-function allele. Such an approach was successfully applied by Kelvin Wong, who isolated a promising suppressor that not only rescues the lethality but also the engulfment defect of *ced-10(ts1875)* mutant worms (K. Wong and M. O. Hengartner, *personal communication)*. However, further analyses are required to reveal the molecular nature of this suppressor.

Summarising the existing data, there is no doubt that further engulfment genes must function in the engulfment of apoptotic cells in *C. elegans*.

However, it appears unlikely that these genes can be identified in standard engulfment screens, as such screens have been done close to saturation. Rather, alternative forward or reverse genetic screens must be performed. Alternatively, to avoid embryonic lethality, a genome-wide RNAi screen could be performed. Since worms can be put on RNAi plates as young larvae, the effect of RNAi on engulfment could be analysed in the germ line of adult hermaphrodites, thereby circumventing embryonic lethality. Another common drawback of conventional screens is their disability to uncover redundant genes. Thus, to avoid redundancy, future engulfment screens could be performed in a sensitive genetic background.

Generally, the main burden of any engulfment screen is the circumstance that unengulfed cell corpses can only be detected by Nomarski microscopy. Developing strong fluorescent markers that allow the detection of unengulfed cell corpses under a dissection microscope equipped with epifluorescence might therefore be the main challenge for the development of successful engulfment screens.

5.2. Deciphering the Biochemical Nature of the Engulfment Machinery

Further elucidation of the engulfment machinery does not only require the genetic identification of novel players, but also the detailed molecular characterisation of already known engulfment genes. Most of today's knowledge of the *C. elegans* engulfment machinery has been derived from genetic studies and only recently, researchers have begun to elucidate the biochemical properties of the proteins participating in the engulfment process. The receptor CED-1 and the adaptor protein CED-6 have been found to interact in vitro [6] and the mammalian homologues of CED-5 and CED-12, Dock180 and Elmo, have been shown to facilitate the exchange of GTP for GDP on Rac, raising the intriguing possibility that CED-5 and CED-12 act together as an unconventional GEF in *C. elegans* as well [14,15]. However, little is known about the biochemical characteristics of CED-7, which represents

the only protein of the engulfment machinery that must act in both the engulfing and the apoptotic cell. Thus, analysing the different domains of CED-7 could provide new insights into its particular function in the engulfment of apoptotic cells. Furthermore, knowledge and comparison of the subcellular localisation pattern of CED-2, CED-5 or CED-6 in wild-type as well as engulfment defective cells should provide further insights into the in vivo function of these molecules.

Data presented in this thesis strongly suggest that apoptotic cells in *C. elegans* expose PS as well. This finding demonstrates that labelling of apoptotic cells by PS is evolutionarily conserved and provides a challenging starting point for future engulfment studies. One of the main unsolved issues in the field of phagocytic research is the mechanism of PS translocation across the plasma membrane. Which proteins are responsible for the transport of PS from the inner to the outer membrane leaflet of the apoptotic cell? Although multiple investigators have addressed this question, the existing data remain conflicting and incomplete. One of the proteins that has been proposed to be involved in PS exposure in mammalian cells is the phospholipid scramblase PLSCR1[16-18]. However, cells derived from PLSCR1$^{-/-}$ mice are not defective in PS exposure, querying the proposed role of PLSCR1 as a scramblase[19]. To shed some light into the mechanisms of PS exposure, further studies can now be performed in the simple but sophisticated worm *C. elegans*.

One attempt to identify the genetic players involved in PS exposure has been described in Chapter 4. Ablating the function of *tat-1*, which encodes the worm homologue of yeast Drs2p, a potential amino-phospholipid translocase implicated in endosomal/vesicular protein transport[20-22], by RNAi impairs PS exposure and causes a moderate engulfment defect. However, the biochemical function of TAT-1 in PS exposure has not been resolved yet. Additional studies, such as the determination of the subcellular localisation pattern, should shed some light on the molecular function of TAT-1 in PS exposure. Likewise, the function of ATPase II, the mammalian homologue of TAT-1, should be analysed further. Does mammalian ATPase II display

distinct functions in healthy versus apoptotic cells? It might be possible that ATPase II behaves as an inwardly directed translocase in healthy cells whereas it acts as an outwardly directed translocase in dying cells. To test this possibility, mammalian cell culture experiments could be performed, measuring PS exposure on apoptotic cells in which the function of ATPase II has been knocked out by RNAi.

The continuous development of novel technologies, such as RNAi or microarrays, and the improvement of established experimental methodologies, such as the automation of yeast two-hybrid screens or the generation of deletion mutants by reverse genetics, provide robust tools for further excursions into the fascinating world of programmed cell death and engulfment. Thus, it is most likely that further exciting discoveries will be made in the nematode *C. elegans*, having strong impacts not only in worms but also in mammals.

5.3. References

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